Nutritional Status and Trophic Dynamics of the Norway Lobster *Nephrops norvegicus* (L.)

by

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Author's Declaration

I hereby declare that I am the sole author of the work contained within this thesis and performed all of the work presented, with the following exceptions;

Stable Isotope analysis- Chapter 2 (2.2.2.7) and 6 (6.2.3)
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All marine species names
were checked for current validity
via WoRMS
World Register of Marine Species
www.marinespecies.org
When a name has changed the new name
will appear in the text with the named
given in the cited publication in parenthesis.
e.g. *Marsupenaeus (*as *Penaeus) japonicus*
all names correct as of 10th February 2012

Abstract

Nephrops norvegicus is a mid-sized benthic decapod lobster found at depths of between 30-300 metres along the eastern Atlantic coast from Norway to Morocco, and within the Mediterranean sea. They inhabit marine muddy sediments in which they excavate burrow systems and from which they make short excursions to feed by predation and scavenging. The females of this species are known to reside within their burrows over the winter period while brooding their eggs, although their feeding behaviour over this period has not been investigated.

Two aspects of the nutrition of *N. norvegicus*, namely nutritional status (the condition of an animal, which is maintained through a balance between energy intake and energy expenditure) and trophic dynamics (the flow of energy from exogenous inputs throughout food webs in an ecosystem) have been studied, to answer questions relating to the influence of season, sex and site on nutrition in this species. Specific questions that have been addressed include: with regard to season, is there a reduction in the nutritional status of *N. norvegicus* over the winter due to a decrease in primary production? With regard to sex, do females have a reduced nutritional status to males when they emerge from their winter brooding period, indicating that females go through a period of fasting over the winter? Also do females employ a filter feeding strategy over the winter? With regard to site, do *N. norvegicus* at two different sites on the west coast of Scotland (i.e. the Clyde Sea Area [CSA] and the North Minch [NM]) occupy different trophic levels and have different fatty acid signatures, which would indicate that their diets are made up of different prey species?

A range of biochemical markers was tested to describe how *N. norvegicus* reacts to a period of forced starvation, and thus to determine their nutritional status. There was clear metabolic depression, indicated by an increase in the copper concentration of the hepatopancreas in both males and females. In contrast to the findings of previous studies, lipids in the hepatopancreas did decrease with starvation, however was mitigated by the metabolic depression and only became significantly lower than fed individuals after 12 weeks in males and 20 weeks in females. Lipids and water combined made up 80% of the mass of the hepatopancreas, and when the lipids decreased the proportional mass was

replaced by water. However, as indicated by the hepatosomatic index (HSI), the total mass of the hepatopancreas decreased over the period of starvation. A temperature effect was also seen which influenced the amount of reserves required by the animal under these conditions.

Using the results from the starvation trials, a predictive tool was developed for determining the nutritional status in *N. norvegicus* from the wild, and for calculating threshold values which indicate whether animals were in a starved state.

These measurements and threshold values were applied to field caught animals, assessing the factors of season, sex and site. The seasonal analysis showed that there was low nutritional status in *N. norvegicus* during the winter of 2008-2009, whereas the nutritional status of winter 2009-2010 was not as low. The site analysis showed that *N. norvegicus* in the CSA, where burrow density is 0.85 m^{-2}, were found to have a lower nutritional status than *N. norvegicus* in the NM, where burrow density was only 0.55 m^{-2}. These results are consistent with the notion that in high density areas *N. norvegicus* has a lower nutritional status than in low density areas.

Females were found to have a larger lipid store within the hepatopancreas than males, presumably to sustain reproduction. Females were also found to be metabolically depressed over the winter and spring months of early 2009. However they were not in an advanced state of starvation over this period. It is therefore concluded that over the winter months females reduce feeding, perhaps in a response to reduced mobility brought about by the brooding stage of reproduction, but do not follow different feeding strategies to males over this time.

The differences of trophic level between *N. norvegicus* in the CSA and the NM were assessed through the use of stable isotope analysis. It was found that although the animals in the CSA had higher $\delta^{15}N$ values than the animals in the NM, the animals in the NM were actually feeding at 0.6 trophic level units higher than those in the CSA. The high $\delta^{15}N$ values in the CSA were ascribed to organic enrichment of nitrates in that area. There was no significant difference between males and females at the end of the winter period, indicating that when females

are brooding their eggs they do not use different feeding strategies (e.g. filter feeding) as an alternative means of nutrition.

The differences between the Fatty Acid (FA) signatures *of N. norvegicus* in the CSA and the NM were determined by fatty acid analysis. A clear difference was demonstrated between sites, indicative of differences in the dietary composition of the two populations, and suggestive of a greater input from pelagic food sources (either zooplankton or fish) in the NM. However, no significant difference was found in FA signatures between male and female *N. norvegicus* at either site or at any time of the year. Thus, consistent with the conclusions from the other monitoring methods, the FA signatures indicate that females and males had similar feeding patterns at all times of year, with regard to the type of food consumed.

As well as addressing issues directly concerned with the nutrition of *N. norvegicus* in relation to season, sex and site, the results of these studies have a number of wider applications, including the development of optimal feeding strategies for holding lobsters under impoundment conditions for long periods, and for identifying the procedures required to assess more directly the dietary composition of *N. norvegicus* populations, and especially the contribution of fish to their diet.

Abbreviations

A		
	ANOSIM	Analysis of similarity
	ATL	Approximate Trophic Lever
B		
	BCA	bicinchoninic acid
	BHT	butylated hydroxytoluene
	bpm	beats per minute
C		
	C:M	chloroform/methanol mixture
	C:N ratio	Carbon: nitrogen ratio
	CL	Carapace length
	Cl:Wt	Carapace length : weight ratio
	CSA	Clyde Sea Area
	CTP	Critical Time Point
D		
	DAM	Deep Abductor Muscle (tail muscle)
	DMA	Dimethyl aldehyde- Fatty acid
E		
F		
	FA	Fatty Acid
	FAMEs	Fatty acid methyl esters
G		
	GCA	Gut Content Analysis
	GLM	General Liner Model
	GSI	Gonadosomatic index
H		
	Haem.	Haemolymph
	Hp	Hepatopancreas
	HSI	Hepatosomatic index
I		
	IAEA	International Atomic Energy Agency
	ICES	International Council for Exploration of the Sea
J		
K L		
M		
	MD	Metabolic Depression
	MD_i	**I**ntrinsic Metabolic Depression
	MD_t	Temperature Dependant Metabolic Depression
	MDS	Multi Dimensional Scaling
	MFV	Marine Fishing Vessel
	MUFA	Monounsaturated Fatty Acid
N		
	NM	North Minch
	NMID	Non-Methylene Interrupted Fatty Acid

O

O:N ratio	Oxygen : Nitrogen ratio
OFN	Oxygen free nitrogen

P

PNR	point of no return
POM	particulate organic matter
PUFA	polyunsaturated fatty acids

Q

R

RV	Research vessel

S

SD	Standard deviation
SE	Standard error
SFA	Saturated Fatty Acid
SIA	Stable Isotope Analysis
SOM	Size onset Maturity
ST	Starvation threshold
SUERC	Scottish Universities Environmental Research Centre

T

TEF	Trophic enrichment factor
TL	Trophic Level
TLC	Thin Layer Chromatography

U

UMBSM	University Marine Biological Station Millport

V

W

Wt	Weight

X Y Z

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1 General Introduction

1.1 Nutrition

Nutrition is a major foundation of life, and a food supply is required by all living organisms to survive, grow and reproduce. The need for nutrients is universal and in that way affects every aspect of biology from the molecular level (determining the reaction of the animal to food presence or absence) to the ecosystem level (through a diversity of different organisms which interact creating whole ecosystems based around nutrient supply). Through evolution and the passage of time the different types of food supply have driven changes in the morphology, physiology and behaviour of all organisms. From the earliest evolved single-celled organisms phagocytosing their food, through to the specialised jaws and digestive systems of carnivorous mammals, all have to deal with which food is available.

Two important aspects of nutrition are nutritional status and trophic dynamics and these have been the focus of the work reported in this thesis.

1.1.1 Nutritional status

Nutritional status is the condition of an animal, which is maintained through a balance between energy intake and energy expenditure. Hu *et al.* (2011) state that this balance of the two processes will determine the scope for growth. Nutritional status has been used to assess human health, with the extremes being severe malnourishment (where energy expenditure far exceeds energy intake) and morbid obesity (where energy intake far exceeds energy expenditure). It can be measured via food ingestion (energy intake) and oxygen consumption (energy expenditure).

In the natural environment the nutritional status of an animal can fluctuate for a number of reasons, including food availability, a change in behaviour due to predation risk (Macleod *et al.*, 2008), or other periodical behavioural traits that remove the animal from available food. Animals have created coping mechanisms to deal with periods without food, which range from reducing their

metabolic rate to varying extents through to complete torpor utilising reserves such as fat for energy (Roots, 2006).

1.1.1.1 Starvation and fasting

The nutritional status of an animal is strongly influenced by starvation or fasting, which are two related but distinct processes. The term 'starvation' refers to the biological condition in which an animal is deprived of food when it is otherwise willing to eat, whereas the term 'fasting' refers to the condition in which an animal is able to eat but for some reason chooses not to do so, due to some intrinsic mechanism such as predator avoidance, ecdysis or behaviour such as reproduction (McCue, 2010). That author considers that the responses of an animal to starvation (the 'starvation syndrome' McCue, 2010) involve a series of integrated physiological processes, in two main phases 'metabolic depression' and the utilisation of 'primary metabolic fuels'.

1.1.1.2 Metabolic depression – Phase 1 of response to starvation

In response to starvation, animals depress the rate of metabolic processes in their body, thus reducing the energy they expend. In this way vital metabolic reserves are maintained, and survival time is extended when conditions are unfavourable for normal life. Throughout the episode, however, metabolism must still be sustained by metabolic reserves, albeit in low amounts (and the problem of accumulated metabolic wastes must be addressed to prevent self-poisoning).

Depression of metabolic rate also occurs in response to other stresses such as temperature change, anaerobiosis, aridity and osmotic changes. It has been recorded in most animal phyla (Guppy & Withers, 1999), and can vary in extent from almost total suppression of metabolic activity (as in diapause) to mild forms of depression (as in various levels of torpor). The occurrence of diapause in crustacean species, related to animal size and reproductive life span, has been reviewed by Hairston & Caceres (1996); malacostracans have long reproductive life spans and little or no occurrence of diapause (Alekseev & Starobogatov, 1996).

Extreme suppression of metabolism involves the almost complete cessation of metabolic activity (cryptobiosis) and represents a survival response to either extremely low temperatures (cryobiosis), which can involve a non-lethal partial freezing of the animal), hypoxia (anoxybiosis) due to lack of oxygen, desiccation (hydrobiosis) due to drying up ponds, or changes in osmotic pressure (osmobiosis) due to high solute/salinity concentrations. All of these types of metabolic depression lead to a severe reduction in metabolic rate, which can nevertheless return to normal levels when more favourable environmental conditions return, allowing the animal to resume its normal activities. These extreme forms of metabolic depression are beyond the scope of this thesis, but have been reviewed by Wright (2001).

Animals which display less extreme forms of non-cryptobiotic responses to metabolic challenges (e.g. torpor) express two forms of metabolic depression, namely intrinsic metabolic depression (MD_i), caused for example by a lack of food, and extrinsic metabolic depression, due for example to a reduction in (non-freezing) temperature (MD_t).

Intrinsic metabolic depression (MD_i) occurs in many animals from a number of different phyla (Guppy & Withers, 1999), including a number of decapod crustacean species. The brown shrimp *Crangon crangon* displays a 10% fall in oxygen consumption for the first 10 days and a total of 42% fall over the full 30 days of starvation (Regnault *et al.*, 1981). The penaeid shrimp *Fenneropenaeus chinensis* deprived of food decreases its oxygen consumption over the first 2 days and then remains at a steady consumption rate thereafter (Zhang *et al.*, 2009). The false southern king crab *Paralomis granulosa* decreases its oxygen consumption for the first 9 weeks of starvation (Comoglio *et al.*, 2005). The necrophagous Antarctic isopod *Waldeckia obesa* decreases its oxygen consumption over 60 days of starvation, with an increase seen at day 25 followed by a further decrease (Chapelle *et al.*, 1994). The protrandric spot shrimp *Pandalus platycerous*, however, maintains the same oxygen consumption for 61 days of starvation before the rate declines (Whyte *et al.*, 1986). Powell & Watts (2010) showed that different crayfish species of the genus *Procambarus* show different extents of metabolic depression when deprived of food; *P. zonangulus* decreases its oxygen consumption significantly, whereas *P. clarkii* does not.

As can be seen from the above examples, there is great interspecies variation in MD_i among the crustaceans, and therefore generalisations are not possible. Rather, when considering a particular species it is important to establish the effects of nutritional stress on MD_i directly, by appropriate measurements.

Metabolic depression in animals can also be induced by other factors such as a fall in temperature (MD_t). In such cases (excluding the effects of freezing) the depression of metabolic rate most likely occurs according to the Q_{10} effect, resulting in a 2-3 fold decrease for every 10°C drop in temperature (Guppy & Withers, 1999). The yabby crayfish *Cherax destrutor* is reported to enter a state of torpor when the water temperature drops below 16°C, causing the metabolic rate, feeding and growth to virtually cease (Withnall, 2000). However, Jones & Obst (2000) showed that when this temperature effect is combined with starvation, *C. destrutor* saves more nutrient reserves than when held starved at a higher temperature, indicating that MD_i is influenced by MD_t. The converse, that the effect of MD_t is influenced by MD_i, may also hold, since in a starvation experiment on the shore crab *Carcinus maenas*, Newell & Bayne (1973) showed that the metabolic depression due to the lowering of the water temperature was less in unfed animals than in fed individuals.

Variation in MD_i can occur to different extents in closely related species. Hervant *et al.* (1999) showed that two subterranean amphipods (*Niphargus rhenorhodanensis* and *N. virei*) can reduce oxygen consumption to lower active levels than can a similar species (*Gammarus fossarum*) which is surface dwelling. This suggests that the subterranean amphipods have lower energetic requirements, and are thus better adapted to long term starvation, than surface dwelling species.

Metabolic depression has been measured in several different ways in previous studies. The classical approach involves measuring the change in oxygen consumption of the animal. Thus Hu *et al.* (2011) used oxygen consumption to assess the metabolic depression due to starvation in the Asian horseshoe crabs *Tachypleus tridentatus* and *Carcinoscorpius rotundicauda*. Brito *et al.* (2010) consider this method, which is also known as indirect calorimetry, to be the best method to estimate basal metabolic rate.

Direct calorimetry measures the actual energy being used by the animal; this is more accurate than monitoring oxygen consumption, since different metabolic reserves will generate different amounts of energy while the animal maintains the same oxygen consumption. Van Ginneken & Van den Thillort (2009) demonstrated how direct calorimetry could be applied to small fish and aquatic crustaceans.

It is not always possible to measure the metabolic rates of large groups of individual animals sampled from a natural population, due to practical limitations, or in some cases due to the fact that the act of capture itself leads to acute changes in the physiology of the animals, or sometimes to their death. For this purpose it is therefore necessary to apply suitable proxy measures of the animals' underlying metabolic rate that can be applied post-mortem.

As Storey & Storey (1990) demonstrated, metabolic depression is modulated via modification of particular regulatory enzymes, and measurements of the relative activities of these enzymes can therefore be used as proxy measures for metabolic depression. Dall (1981) found that the amount of haemocyanin (the oxygen carrier protein) decreases in the haemolymph of crustaceans in conjunction with such reductions in metabolic rate (reviewed by Depledge & Bjerregaard, 1989). Since each haemocyanin molecule contains an active site with two copper atoms, measuring the concentration of copper in the haemolymph offers an indirect way of estimating the amount of haemocyanin, as copper does not exist in appreciable quantities in other forms in the haemolymph (Taylor & Anstiss, 1999). This indirect measure can also establish the fate of the copper atoms released when the haemocyanin molecules are broken down, an approach used by Spoek (1974). Taylor & Anstiss (1999) suggested that the principal site for the accumulation of copper released in this way is the hepatopancreas. Therefore when the concentration of copper in the haemolymph decreases, it would be expected to increase in the hepatopancreas in a complementary manner. Measurements of the concentrations made simultaneously on the hepatopancreas and the haemolymph therefore provide a powerful way in which to establish if this is the case.

1.1.1.3 Utilisation of reserves – Phase 2 of response to starvation

Metabolic depression leads to a new balanced steady state in which there is a general reduction of the rates of all cellular processes. However, in conjunction with this phase of the starvation response (Phase 1), during a period of starvation selected adjustments also occur in the utilisation of fuel reserves (Phase 2). The latter includes catabolism (oxidation) of carbohydrates, lipids, protein, and their metabolic conversion into different forms (of amino acids in proteins, or in fatty acids in lipids). Auerswald *et al.* (2009) reviewed the catabolic pathways of proteins and glycogen that produce pyruvate, which is then converted to acetyl-CoA (which is also produced directly by lipids through β-oxidation of fatty acids). Acetyl-CoA then enters the tricarboxylic acid (TCA) cycle and adenosine triphosphate (ATP) is produced from adenosine diphosphate (ADP). Proteins also enter the TCA cycle via oxoglutarate and α-ketoglutarate stages. Study of the enzymes that produce these intermediate metabolites could yield vital information concerning which resource is being used.

Ritar *et al.*(2003) suggested there are three distinct stages within the resource utilisation period in crustaceans (i.e. Phase 2), with lipids from stores being utilised first, followed by protein catabolism and then the breakdown of membrane phospholipids prior to death. However, Sanchez-Paz *et al.* (2006) emphasised the diversity of responses by different crustacean species to starvation, suggesting that there is no one type of physiological resource that is used preferentially in all crustaceans during starvation. Some species such as *Marsupenaeus (*as *Penaeus) japonicus (*Cuzon *et al.*, 1980) will utilise glycogen first, while other species such as *Hemigrapsus nudus* (Neiland & Scheer, 1953) will utilise protein first, and yet other species such as *Niphargus virei* (Hervant *et al.*, 1999) will utilise lipids first.

The result of Phase 2 is that there is a mass change in the organism either as whole body weight or organ-specific weight. McCue has summarised the extensive use of this measure in studies of starvation in birds, mammals, reptiles and fish (McCue, 2010, Table 2). However, in decapod crustaceans there is a difficulty in this. When decapods reduce resources from their tissues then water is taken up to maintain the necessary body volume and internal turgidity

(Comogilo *et al.*, 2005). This mass equalisation negates the usefulness of any whole body measurements. However, organ-specific weights can be taken instead and there is some evidence that there is functional prioritisation of specific organs in an animal's response to starvation (McCue, 2010).

It is unlikely that Phase 1 and Phase 2 will occur independently, but rather that there is a continuous series of physiological responses (McCue, 2010). The measurable interplay between phase 1 and phase 2 is species specific for example *Paralomis granulosa* as measured by Comoglio *et al.* (2005), showed a drop in oxygen consumption for the first 9 weeks, followed by utilisation of lipids. Whereas the reaction to starvation of *Fenneropenaeus chinensis* as measured by Zhang *et al.* (2009), showed a small initial reduction of oxygen concentration for the first four days followed by an increased O:N ratio (which is indicative of lipid catabolism). The southern king crab *Lithodes santolla,* as measured by Comoglio *et al.* (2008), showed no alteration in oxygen consumption, but only the physiological resource utilisation phase, with protein used as the primary reserve throughout the experiment.

This short review of the responses of crustaceans to starvation highlights the need prior to any field study of the nutritional status of a particular species of crustacean, to establish the species-specific response to starvation, in a controlled aquarium based starvation study. In undertaking a study of the nutritional status of the Norway lobster, *Nephrops norvegicus*, which is one of the main aims of the scientific investigation reported in this thesis, as introduced in Section 1.3, this approach has therefore been followed. The extent of existing knowledge of starvation in *N. norvegicus* is discussed in Chapter 2.

1.1.2 Trophic dynamics

The concept of 'Trophic Dynamics' was first outlined by Lindeman (1942), who coined this term to describe the flow of energy from exogenous inputs throughout food webs in an ecosystem. This concept refers to the quantitative and qualitative study of how organisms interact as predators and as prey. Trophic dynamics are important for an understanding of ecosystem health since

differences in feeding in relation to habitat, season and even sex, can be indicators of differing life history strategies in a species.

Lindeman (1942) stated that energy from prey organisms will pass to the consumer organisms through discrete trophic levels (TL), where the consumer is at one trophic level higher than the prey organism. Therefore he envisaged discrete trophic levels, where TL 1 is defined as a primary producer, TL 2 a first order consumer (herbivore), TL 3 a predator of herbivores and so on. Odum & Held (1975), however, stated that most species consume prey items at more than one trophic level. Therefore they proposed a system that used the proportion of the diet (observed through gut contents) to generate fractional trophic levels. Later Adams (1983) formulated this into the following equation:

$$\tau_i = 1.0 + \sum_{j=1}^{n} \tau_j \, (F_{ij}) \qquad \text{Equation 1.1}$$

Where:

τ_i= Trophic level of species i

τ_j= Trophic level of prey species j

$F_{ij=}$ Fraction of the consumed food (volume) of species i consisting of food category j

Adams gave a number of examples of this formulation, including for the large mouth bass. The diet of this species is known to consist of 50% threadfish (*Nemichthys scolopaceus*), gizzard shad (*Dorosoma cepedianum*) (TL=1.49), 25% sunfish (*Lepomis sp.*) (TL=2.1) and 25% freshwater drum (*Aplodinotus gruniens*) (TL 2.3). Therefore applying these values to the above equation it was found that the large mouth bass was feeding at a TL of 2.85:

1 + 1.49 (0.50) + 2.1 (0.25) + 2.3 (0.25) = 2.85.

This equation is still used today in trophic level based modelling programs such as EcoTroph (Gascuel *et al.*, 2011). Therefore, to determine the fractional TL of the study species in this way, all the food items need to be identified and quantified. This has been attempted previously for *N. norvegicus*, as Thomas & Davidson (1962) recorded gut contents of this species from the Clyde Sea Area,

Minch, Moray Firth and the Firth of Forth. Parslow-Williams *et al.* (2002) also performed gut content analysis on this species. These studies suggest that the diet of *N. norvegicus* is comprised of many species from various phyla, including crustaceans, echinoderms, polychaetes and molluscs. These studies, however, have a notable limitation due to their primary method of prey detection being gut contents analysis. As both Yonge (1924) and Oakley (1978) pointed out, this method over-estimates the quantity of food items with hard calcareous structures, and as Yonge states it was 'often difficult or impossible to identify the fragments, due to effective masticatory action of the mouth parts.' Therefore obtaining a complete picture of 1) the full prey spectrum and 2) the relative proportions of these in the diet is very problematic. Newer methods need to be employed to determine the trophic dynamics of *N. norvegicus*.

As already mentioned, Lindeman (1942) stated that energy is transferred through the various trophic levels. However, along with the transfer of energy between trophic levels, carbon and nitrogen are incorporated into the tissues when lipids and proteins are synthesized from the diet (Parker *et al.*, 2005). Carbon enters the base of the food web via CO_2 to be utilised in photosynthesis. There are two stable isotopes of carbon of interest within living organisms, the heaver (^{13}C) isotope and the lighter (^{12}C) isotope. In general ^{13}C is preferentially used and then expelled following photosynthesis, and so the ratio of ^{13}C:^{12}C will be lower in carbon fixed in plant tissues than in the atmosphere. Plants can be categorised into three types, according to the particular photosynthetic pathway that they employ (C_3 Plants use the Calvin cycle, C_4 plants use the Hatch-Slack cycle and other plants use Crassulacean Acid Metabolism). Each pathway uses the isotopes in different proportions, with the result that the ratio of ^{13}C:^{12}C will be less in C_3 plants than in C_4 plants (as reviewed in Marshall *et al.*, 2007). Marine primary producers use the photosynthetic C_3 pathway; that said, they differ from terrestrial primary producers which use the C_3 pathway, due to the different sources of dissolved carbon used by marine primary producers. Boulton (1991) showed that phytoplankton have a ratio of ^{13}C:^{12}C that is much lower than that of macroalgae from shallower water. These carbon isotope ratios can therefore be used to determine the source of organic material entering the food web.

Two nitrogen isotopes of interest, which make up the predominately protein-based nitrogen pool within an organism, are ^{14}N and ^{15}N. The lighter isotope (^{14}N) is preferentially involved in enzymatic reactions compared to the heavier isotope (^{15}N), and therefore ^{14}N is preferentially excreted by the body (Parker *et al.*, 2005). For this reason the proportion of ^{15}N in the body is greater than that of the source (the food consumed). As amino acids are passed from one organism to another, the enrichment of ^{15}N increases, and for this reason the ratio of ^{15}N:^{14}N reflects the trophic level of the organism. Stable isotope analysis is based on the ratios of both ^{13}C:^{12}C and ^{15}N:^{14}N, as will be discussed in more detail in Chapter 6.

The lipids that are transferred from predator to prey release fatty acids while being digested. Most of these are not catabolised, but are stored in fat stores such as the adipose tissue of mammals, the fat bodies in insects and the hepatopancreas of decapod crustaceans (Iverson *et al.*, 2003). Therefore the fatty acid profile of an organism can provide evidence of the type of prey it has consumed. How fatty acids are measured, and examples of how they can inform trophic level studies, are described in more detail in Chapter 7.

1.2 *Nephrops norvegicus* (study species)

1.2.1 Introduction to species

Nephrops norvegicus is a mid-sized lobster from the Nephropidae family. It is a sediment-dwelling decapod crustacean that lives in discrete burrows in suitable soft sediments in the seas around Europe.

1.2.2 Anatomy

Like other decapods, *N. norvegicus* (Figure 1.1) possesses a cephalothorax covering its head and thorax with a hard carapace, and an abdomen ('tail'). Five pairs of pereiopods also extend from the thorax, with the first pair being enlarged chelipeds (claws). Yonge (1924), described in some detail the mechanics of feeding from grasping to consuming through to assimilation, and described in depth the anatomy of the digestive system. The mouthparts

comprise a pair of mandibles and two pairs of maxillae, which together with three pairs of maxillipeds (which are strictly thoracic appendages) are involved in detecting and handling food, and passing it to the mouth and thence to the stomach via the oesophagus. In the stomach the gastric mill grinds up the food and then from the foregut it passes through the mid-gut and then to the hind-gut. The carapace encloses the gill chamber on each side, and internally the thorax contains the stomach with gastric mill, the hepatopancreas (which is also the storage organ for lipids and glycogen) and the gonads.

Posterior to the thorax is the abdomen which comprises 6 somites (sections) each of which bears a pair of pleopods (swimmerets), the first (most anterior) of which is modified in the male into thickened structures for the transfer of spermatophores during copulation. This feature can be used to distinguish the sex of the animal. The abdomen terminates with a tail fan comprising a post-segmental telson (the location of the anus) and paired biramous uropods, which are used to aid swimming and occasionally for excavating. *N. norvegicus,* like many other decapod crustaceans with elongated abdomens, has an extensive abdominal musculature comprising sheets of slow-contracting fibres to maintain posture (Neil & Ansell, 1995), and blocks of fast-contracting fibres (Govind, 1995) to produce the rapid and powerful flexions and extensions that generate the tail-flip swimming mechanism (Newland *et al.*, 1992). As in crayfish (Krasne & Wine, 1975), in response to intense mechanical stimuli, giant nerve fibres activate stereotyped tail-flip responses which propel the animal away from the source of stimulation and so allow it to evade predators (Newland *et al.*, 1992).

The circulatory system of lobsters (as reviewed by Martin & Hose, 1995) is typical of decapod crustaceans in being partly open. Haemolymph passes around the body from the heart within up to five arteries, and after capillary-tissue gas exchange it passes to the gills and then collects in open spaces (sinuses) for delivery back to the heart. The haemolymph contains the oxygen-carrying protein haemocyanin in solution, which uses two copper atoms for oxygen binding (in comparison with vertebrate haemoglobin, which contains four iron atoms for the same purpose). Haemocyanin is formed and recycled in the hepatopancreas (Taylor & Anstiss, 1999). As well as carrying oxygen, the haemolymph transmits nutrients, cellular waste, hormones and internal defence molecules, and haemocytes (blood cells) which serve the functions of clotting

(through change in morphology causing cytolysis triggering coagulation), removal of foreign material and assist in post-moult re-hardening (Martin & Hose, 1995).

The hepatopancreas is a twin-lobed mass which appears in a variety of colours from cream through brown to dark brown. This is the largest organ within the cephalothorax (apart from the female gonads at later stages of development- see below). This organ is approximately 30-50% lipid (Rosa & Nunes, 2003) and contains many fatty acids, including a high proportion of Omega [PUFA] 3 (Tsape, 2010).The role of the hepatopancreas in performing the functions of the liver ('hepato') and the pancreas was called into question in the 1970s - 1980s by several authors (van Weel, 1974; Gibson & Barker, 1979; Dall, 1981; Dall & Moriarty,1982), since they found that it did not perform these functions. They suggested that the name 'digestive gland' would be appropriate, being more consistent with its role. However, since that time many authors have continued to use the name hepatopancreas, and therefore for the sake of clarity it will also be called hepatopancreas in this study.

According to Factor (1995) the role of the hepatopancreas is for the synthesis of digestive enzymes, the final digestion of food, absorption of nutrients, storage of inorganic reserves and for lipid and carbohydrate metabolism. These functions are performed by the four types of cells contained within the hepatopancreas (E, R, F and B cells). Firstly the E cells or embryonic cells are created by mitosis and subsequently develop into R (Restzellen) or F (Fibrillenzellen) cells (Dall & Moriarty, 1982). Verri *et al.* (2001) also suggests R/F cells could be produced as an intermediary. The F cells can then be developed into B (Blasenzellen) cells. The R cells store nutrients, the F cells secrete enzymes and presumably when there is an abundance of enzymes B cells store digestive enzymes in large vacuoles. Detailed reviews of these cells and how different nutrients (lipids, carbohydrates and proteins) are digested by the enzymes contained within the hepatopancreas are provided by Gibson & Barker (1979), Dall & Moriarty (1982) and Verri *et al.* (2001).

The gonads of males and females are both paired structures located within the cephalothorax. However, the structures are very different between the sexes. The development of the female gonads (the ovaries) normally occurs annually, and involves a change of colour from white/cream to a dark green prior to the

release of eggs. At these later stages of development, lobes of the ovaries extend into the abdominal cavity. These processes will be discussed later. The male gonads are much smaller structures and each comprises a proximal testis, which lies above the hepatopancreas and a vas deferens where the spermatophore is formed (McQuaid & Briggs, 2004).

1.2.3 Feeding

Yonge (1924) described in some detail the mechanics of feeding, from grasping to consuming through to assimilation, and described in depth the anatomy of the digestive system of *N. norvegicus*. This has been detailed above.

The process of chemoreception was found to detect only dead and decaying food, whereas live prey were taken only when they were detected visually or came into contact with the second pereiopods, suggesting that this species is a scavenger as well as a predator (Oakley, 1978), this is also indicated by its propensity to be caught in baited traps.

It has already been noted that gut content analysis of *N. norvegicus* has been the prime method to determine food consumed by Thomas & Davidson (1962) and by Parslow-Williams *et al.* (2002) indicating crustaceans, echinoderms, polychaetes and molluscs are part of the diet of *N. norvegicus*.

Parslow-Williams (1998) also used a combination of other methods, and introduced the use of RNA: DNA ratios to show nutritional condition, as later published in Parslow-Williams *et al.* (2001), who noted that a number of different authors have used this method to determine nutritional status in different animals. The use of RNA:DNA ratios is based on the fact that the energy required for amino acid formation leads to RNA production, providing a link between RNA and feeding level. As DNA is a constant and an indication of species type, the RNA: DNA ratio could be used. Parslow-Williams *et al.* (2001), however, showed that this cannot definitely show starvation in laboratory experiments, and showed that the RNA: protein ratio was in fact a better indicator of starvation level. Their work, however, only took a snapshot of the areas studied, and no long-term study was performed to ascertain the seasonal dynamics according to the RNA: DNA ratiometric method.

Loo *et al.* (1993) proposed that *N. norvegicus* are capable of filter feeding. They showed that food particles as small as 500-600 µm, when placed in suspension in tanks holding *N. norvegicus*, could be found in their stomachs. These would not be detected in a typical gut content analysis (GCA). The concentration was also shown to decrease over the following six hour period. However, filter feeding is perhaps not the most appropriate term for this behaviour, since the animals were observed to grasp the particles (R.J.A. Atkinson pers. comm.). Also, the extent to which the acquisition of small particles contributes to the overall diet of *N. norvegicus* was not in fact fully established, since nutritional status was estimated from the haemocyanin concentration in the blood, which reflects only the initial nutritional status but may not reflect long-term trends.

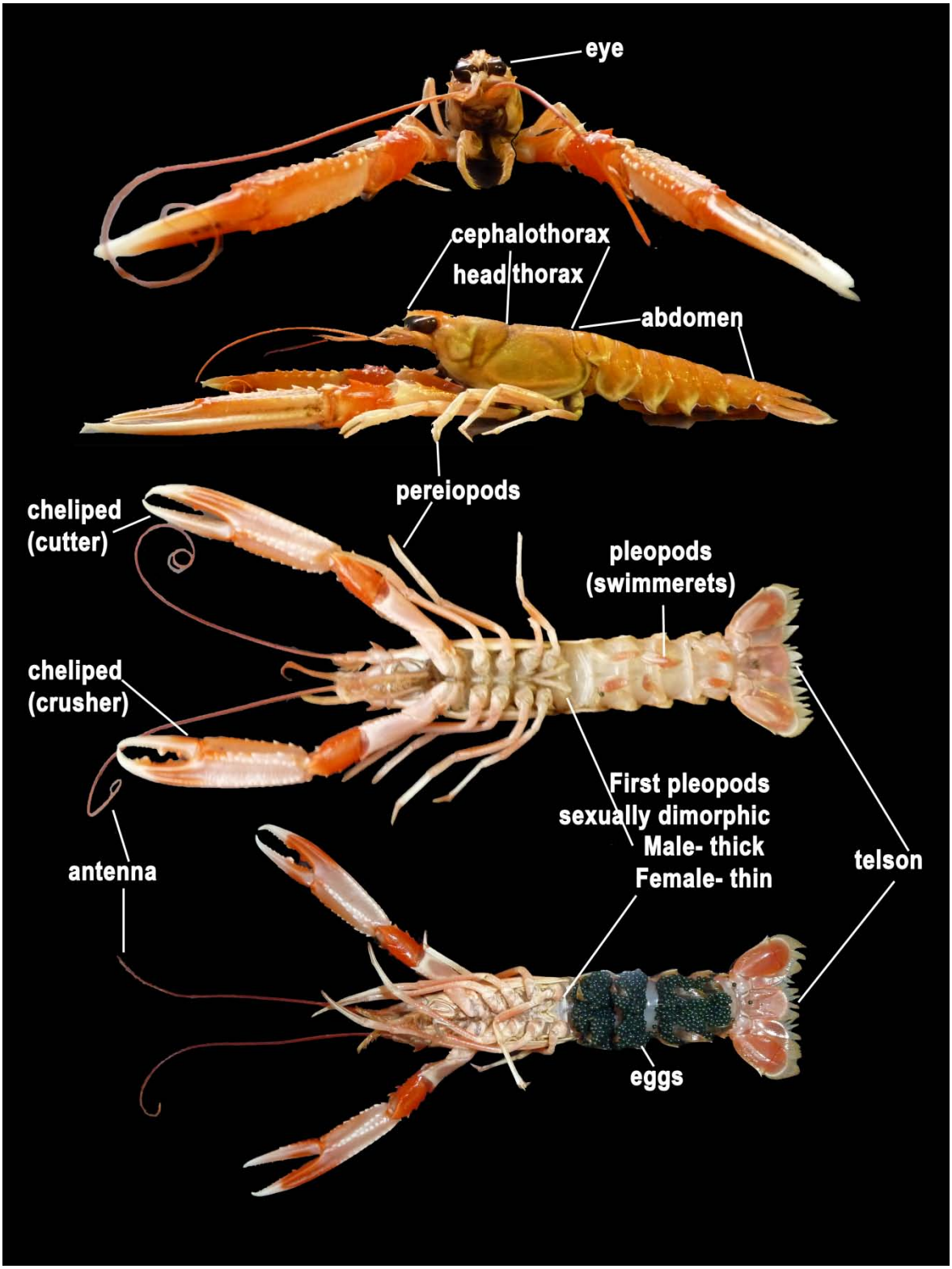

Figure 1.1 *Nephrops norvegicus* anatomy, with the frontal, lateral and ventral view of a male and ovigerous female.

1.2.4 Reproduction

The female reproductive cycle of *N. norvegicus* has been studied in great detail (Bailey, 1984; Mente, *et al.* 2009; Tuck *et al.*, 1997a, 2000). Females become

reproductively mature at around 3 years of age (Bell *et al.,* 2006). As age is not easy to determine, due to the fact that there are no morphological structures that change in an age-related manner and are retained across successive moults, the size of the female is most often used to determine the stage at which it becomes reproductively active. This is known as the 'Size at Onset Maturity' or SOM, which has been defined by Bailey (1984) and Tuck *et al.* (1997a) as the size at which 50% of females (L_{50}) have ovaries in a reproductively-active condition - an indicator of 'physiological maturity' (i.e. at or beyond Stage 3, see Table 1.1). As an alternative indicator of SOM, both Bailey (1984) and Tuck *et al.* (1997a) have also used the size of the smallest ovigerous female -an indicator of 'functional maturity', and found no significant difference between these two methods. A third indicator of SOM is the inflection in relative size of a secondly sexual feature such as abdomen width in females an indicator of morphological maturity.

A large part of the female reproductive cycle involves ovary maturation. The ovaries in sexually mature females will develop from one reproductive cycle to the next, and they go through considerable seasonal changes (Tuck *et al.*, 1997a). Rotllant *et al.* (2005) and Mente *et al.* (2009) described in detail the development of the continuous ovary maturation cycle. Avarre *et al.* (2003) showed that the hepatopancreas was the developmental site of vitellogen (the green pigmented yolk protein) which was then transported to the ovaries via the haemolymph where vitellogen is converted into a number of vitellin products, which are incorporated into the oocyte cells in the ovaries. As these cells develop and increase in size the whole ovary appears green. This progression of the ovary maturation cycle has been determined by a number of authors using a staging method based on the colour of the ovaries (Table 1.1), and the present study has used a variant of this scheme (Figure 1.2). In Stage 1 the ovaries are white and relatively small, but as they develop, they both increase in size (Mente et *al.*, 2009) and become firstly cream in colour (Stage 2), and then pale green (Stages 3-4). The size of the ovaries now increases at a greater rate so that they come to occupy much of the volume of the cephalothorax and can sometimes extend lobes into the proximal segments of the abdomen. Later, as the colour develops to darker shades of green (Stages 5-7) the ovaries become fully mature and the oocytes become distinguishable. Once the eggs are laid and

attach to the pleopods (or are sometimes resorbed) the spent ovaries become white (Stage 8).

Table 1.1 The stages of female ovary maturation, as recognised in various studies. Table adapted from Tuck *et al.* (1997c).

colour	Thomas (1964)	Symonds (1972)	Bailey (1984) and Tuck *et al.* (1997c)	Smith (1987)	This Study	
-	-	0	-	1	-	A
White	0-I	1	0	2	1	
				3		
Cream	II	2	1	4	2	
Very Pale Green	II-III	3	2	5	3	B
Pale green				6	4	
Green	III-IV	4	3	7	5	C
Dark Green					6	
Dark Green-swollen and oocytes distinguishable	V	5	4	8	7	
Reabsorbed/ spent		5R	5	9	8	A

Bailey (1984) summarised the timing of this reproduction cycle as follows. The ovaries in a sexually mature female will develop over the winter period when the animal is still incubating eggs from the previous season. Upon releasing these eggs, in spring to early summer, the female will moult, and whilst its exoskeleton is still soft it is available for copulation with a male. At this stage the ovaries will be pale green or green. If insemination is successful the female will then store the sperm in an external chamber, the spermatheca, also called the thelycum (Aiken & Waddy, 1980), or seminal receptacle (Bauer, 1986), whilst the ovaries continue to mature, reaching Stage 7 by mid to late summer. The eggs are then released from gonopore openings at the base of the third pereiopods, fertilised by the sperm that are mobilised and released from the spermatheca, and attach onto the abdominal pleopods. The ovigerous (so-called 'berried') female will then enter a burrow to incubate the eggs over the winter period. It is for this reason that female *N. norvegicus* are rarely seen in large numbers in winter trawl catches, as reflected in sex ratios biased towards males.

Females will copulate post-moult around early summer in Scotland (Bailey, 1984). If a female has failed to reproduce it will reabsorb the gonads as a mechanism to conserve or recycle its nutrients (Tuck *et al.*, 1997a).

Before the field biology of *N. norvegicus* was understood, Yonge (1924) proposed that females would then migrate further offshore, and that is why the catch of females declined in the winter. This, however, is not the case as they have been found to reside in their burrows while brooding, only to emerge for mating in spring (Chapman & Howard, 1979). These animals are typically found in more complex burrow systems than those occupied by males, with more openings, which may ensure that the eggs are adequately ventilated.

1.2.5 Growth and moulting

Growth in *N. norvegicus* as in all other decapod crustaceans is a discontinuous process involving a series of moults. Increases in size occur immediately after each moulting event (ecdysis) and prior to the re-hardening of the exoskeleton in its intermoult state. The frequencies of these moults differ with age. Bell *et al.* (2006) states that juvenile *N. norvegicus* will moult once per month, which allows them to have a fast growth rate. However, in their 2nd and 3rd year these animals will moult at a slower rate, around 3-4 times a year, and mature male *N. norvegicus* will moult only 1-2 times a year. After the onset of sexual maturity, females grow considerably more slowly than males (Bell *et al.*, 2006), and in these mature females the moulting process is synchronised with their reproductive cycle, and occurs either annually or biennially.

This isometric growth means it is possible to estimate the weight, abdominal length, total length or claw length from the animal's carapace length using regression equations (Farmer, 1974).

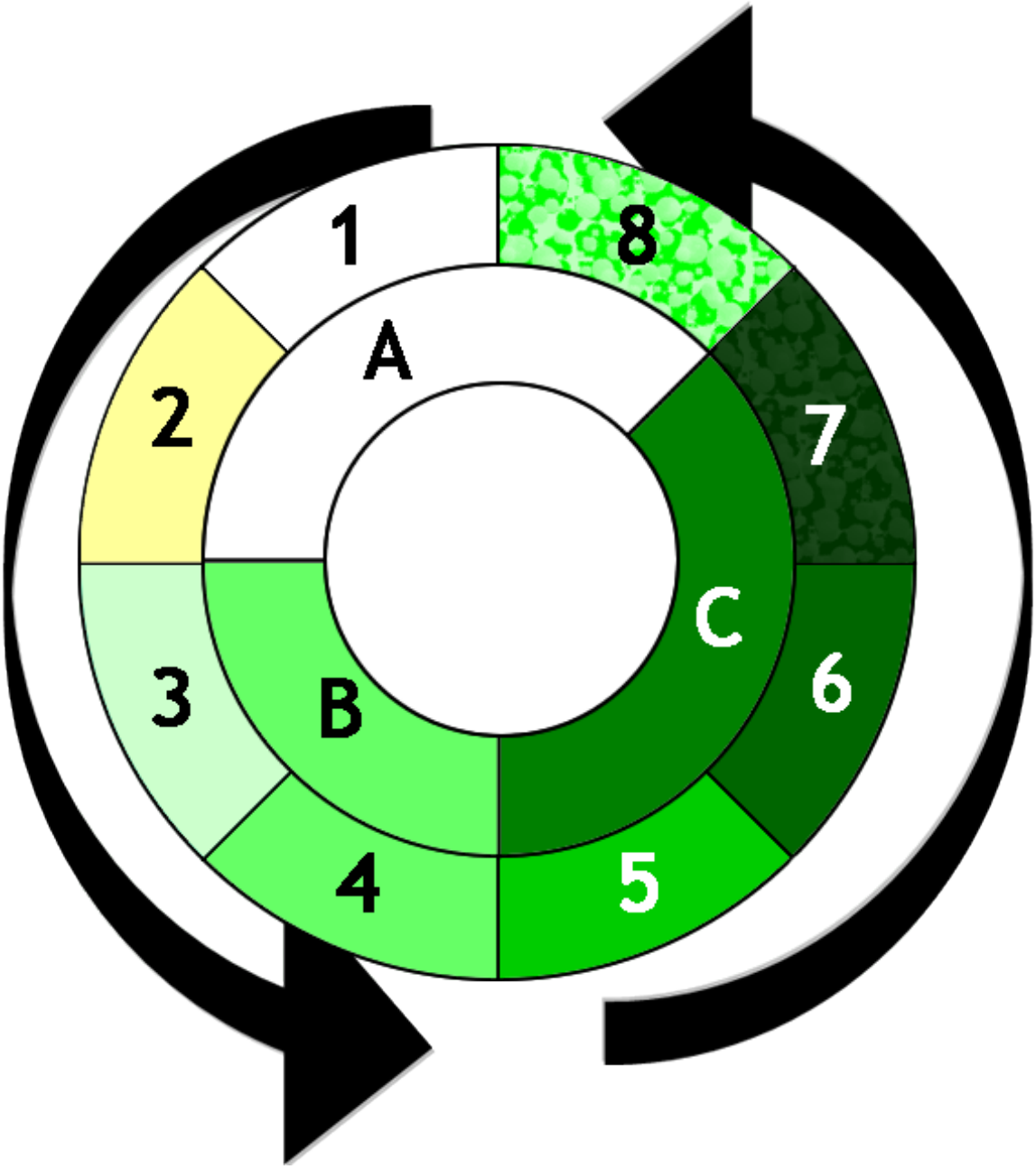

Figure 1.2 Ovary maturation Cycle schematic, showing the circle nature of the cycle, 1- White, 2- Cream, 3-very pale green, 4- pale green, 5- green, 6-dark green, 7- Dark Green- swollen and oocytes distinguishable , 8- spent-reabsorbed (in reality speckled). The letters A, B and C indicate the groupings of these 8 stages. See also Table 1.1.

1.2.6 The geographic range of *N. norvegicus*

N. norvegicus are found from Iceland to Norway in the north west and north east, respectively, to Morocco and elsewhere off north-east Africa and the Mediterranean in the south of its range; see Figure 1.3. They live in depths of between 20-800 metres (sometimes <20 m in Scottish sealochs) in muddy sediment, dwelling in burrows in the sediment and with each animal rarely moving more than a few tens of metres from its home burrow, though animals

may make longer excursions and create new burrows or displace conspecifics (Chapman & Howard, 1979).

Bell *et al.* (2006) summarised a number of different studies that assessed the spawning and hatching periods of female *N. norvegicus* throughout the entire latitudinal range of *N. norvegicus* from the Adriatic Sea in the south to Iceland in the North. They suggested that the period between spawning and hatching is extended at ever higher latitudes until the hatching period overlaps with the following spawning season, driving the population into a biennial breeding cycle. This means that female *N. norvegicus* from Iceland are all in a biennial cycle. Thomas & Figueiredio (1965) noted that 90% of mature females off the west coast of Scotland mated annually. The west coast of Scotland is therefore on the cusp of the shift between annual and biennial reproductive cycling; this could have an effect on this study: if the females studied are in the second year of the cycle, they could have a different nutritional status to animals in a single annual reproductive cycle.

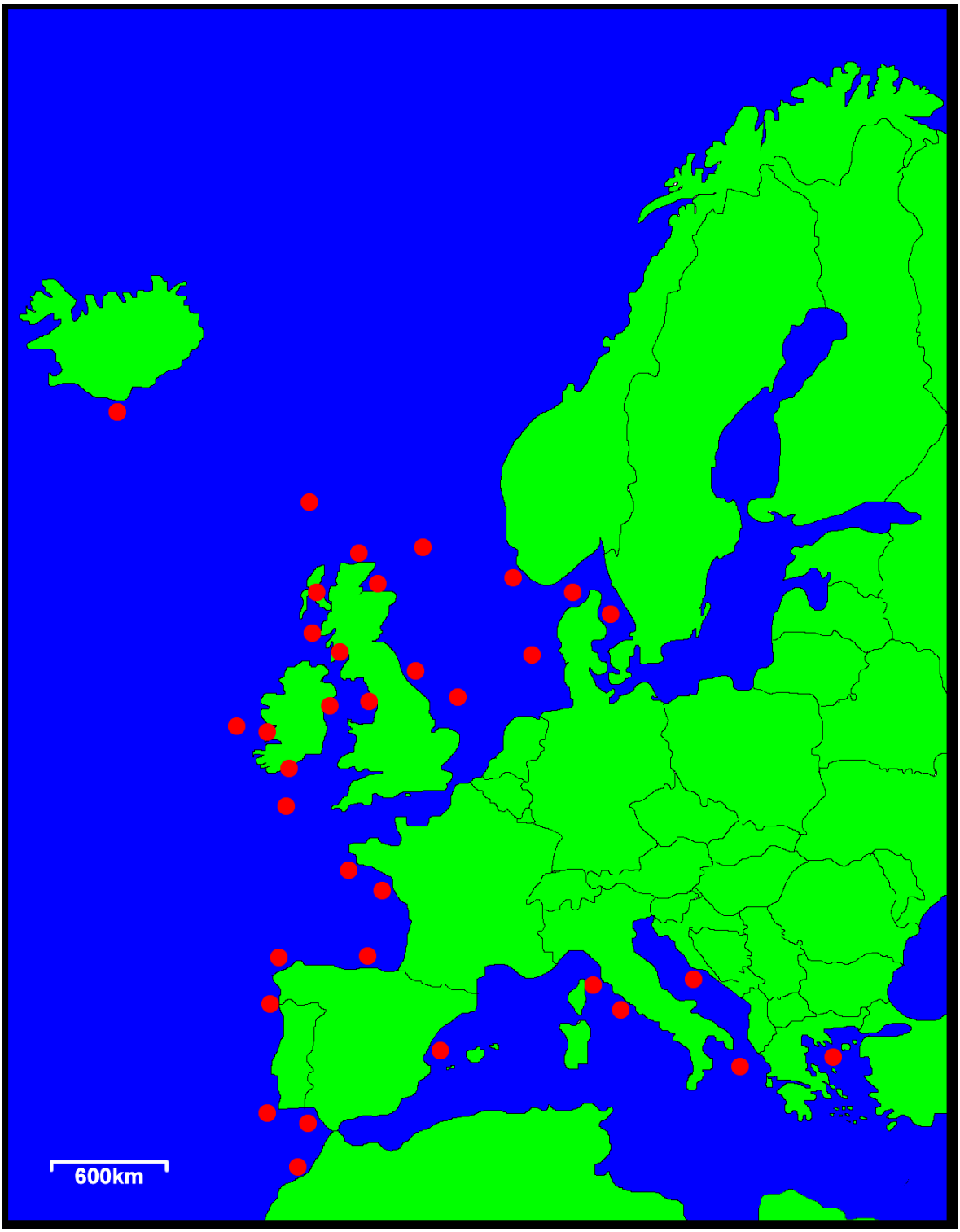

Figure 1.3 Map of the distribution of *N. norvegicus* locations as described in Bell *et al.* (2006). Red circles indicate fishing areas.

1.2.7 Commercial importance of *N. norvegicus*

The commercial importance of *N. norvegicus* grew from a decline in fish stocks, with mixed fisheries increasing the catch of *N. norvegicus* (Bell *et al.*, 2006). 12,741 tonnes of *N. norvegicus* were landed in Division VIa (west of Scotland) in 2009 (ICES, 2011). Figure 1.4 shows the increasing importance of this species relative to the three main whitefish species. The fishery for *N. norvegicus* is closely regulated to ensure it does not suffer the collapses that have occurred in many other fish stocks. The 'Nephrops Working Group' of the International Council for Exploration of the Sea (ICES) closely monitors the size and biometrics of the populations that form the different fished stocks. Increased knowledge about the biology of these animals will contribute to sustaining these stocks throughout their geographical range.

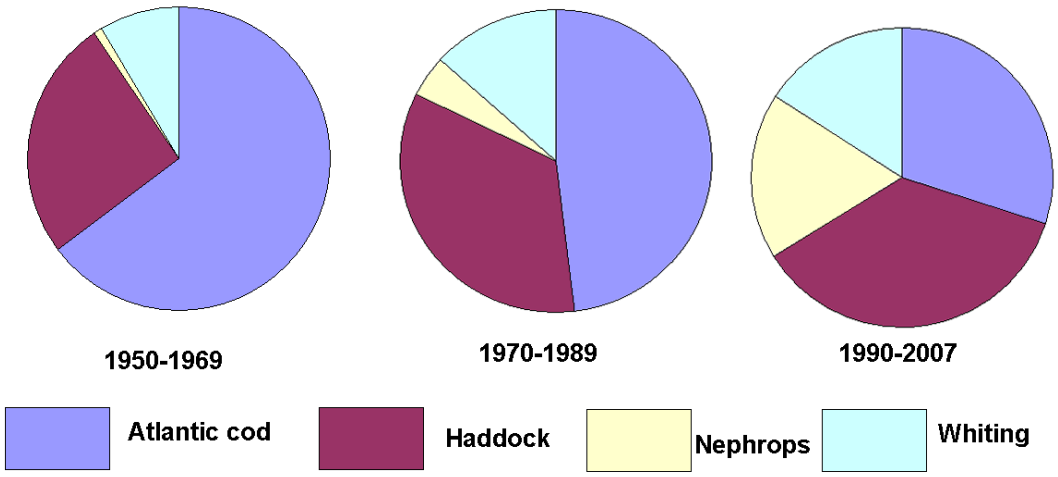

Figure 1.4 Three time point pie charts showing the increase in the importance of the UK *N. norvegicus* fishery compared with cod, haddock and whiting between 1950 and 2007, as percentages of landed weights. (Data source: Fisheries and Aquaculture Information and Statistics Service, 2009)

1.3 Differences in nutritional status

Animals need to balance their food-searching behaviour with the need to avoid predators. *Nephrops norvegicus* is faced with this dilemma; food might not be readily available on the sea bed, but increased searching leads to a greater risk of predation. These conflicting needs are resolved by the animals staying in

close proximity to their burrows during feeding excursions, in order to be better able to evade predators (Chapman & Rice, 1971). Food items need to be encountered within this limited foraging range.

Studies of a wide range of animals have examined the theory that there is a trade-off between starvation and predation risk. For example, in the common starling (*Sturnus vulgaris*) in winter when there is a greater risk of starvation, birds will increase their foraging and accumulate more fat, making them more able to survive the long nights, when feeding is precluded (MacLeod *et al.*, 2008). Also, when there is a greater predation risk the scope of foraging will be reduced and thus the fat reserves will decrease. This occurs for two reasons, firstly because the available energy is limited by the amount of food accessible, and secondly limits weight gain to provide a more effective escape from predators. Bednekoff & Houston (1994) suggested, however, that feeding always outweighs the predation risk, as starvation in birds has more chance of leading to death than the risk of predation. A crustacean such as *N. norvegicus*, being poikilothermic, is not as susceptible to starvation as a bird such as *S. vulgarus*, which needs a considerable proportion of ingested energy for maintaining body temperature and for flight, and indeed *N. norvegicus* can survive for at least 6 months without food. The specific trade-offs that allow this to happen in *N. norvegicus* form another focus of the investigation reported in this thesis.

Many factors can influence the nutritional status of *N. norvegicus*, and three of these, the animal's sex, the time of year and the environmental conditions in which it resides, are reviewed in the following sections.

1.4 Sex-specific differences

A change in behaviour of females occurs as they brood their eggs. Females will increase their time residing in their burrows. This is indicated by the number of males and females in a random sample of a trawl catch (hereafter referred to as the sex ratio) which alter seasonally (in accordance with the maturity cycle) as determined from catch composition. In the winter months the majority of the catch is comprised of males when females are ovigerous. As females remain

within their burrows over this time little is known about their feeding behaviour during this period.

Newland (1985) showed that tail flipping (the method of escape swimming) was suppressed in egg-bearing *N. norvegicus* and that, in these animals, the terminal appendages (uropods) which do not carry eggs and which with the telson form the 'tail fan', are the only significantly flexing sections of the tail. Therefore there would be a greater predation risk for females in leaving their burrows to feed. Aguzzi *et al.* (2007) suggested that females can be attracted out of their burrows when food is available, as indicated by catch of ovigerous females in creels, but then stay within close proximity to a burrow entrance. With this limited foraging range, it is entirely possible that females reduce their feeding rate, or completely cease feeding.

The nutritional status measurements will show whether or not the females which have just emerged from the burrows post-brooding have a reduced nutritional status or not and if this status is significantly different from that of males, which presumably have been able to forage for food all winter. Determining biomarkers of starvation that could be used reliably on field-caught samples would help to answer this question, thus adding to the available evidence about the nutritional status of females over the winter months.

Aguzzi *et al.* (2007) showed that the percentage of females with empty stomachs was significantly higher (60%) when they are ovigerous ('berried') compared with non-ovigerous ('unberried') females (50%). Oakley (1978) also showed that ovigerous females were less likely to react to chemical food stimuli than did either males or non-ovigerous females. Thus 17% of ovigerous females did not react to a chemical stimulus, compared with 5% of males and 10% of non-ovigerous females.

Female *N. norvegicus* could also go through a period of torpor while incubating their eggs in burrows, although there is a constant requirement for them to ventilate and clean the eggs (Waddy *et al.*, 1995).

Parslow-Williams (1998) and Hill (2008) commenting on the paper by Loo *et al.* (1993) suggested that if females were restricted to their burrows during

brooding then suspension feeding could be a strategy that they might use. Trophic level calculations would be a way to determine if this was the case. If they are suspension feeding then their trophic level will be lowered to that of a filter feeder such as the queen scallop (*Aequipecten opercularis*), which has a trophic level of 2.5 (Jennings & Warr, 2003). This will be considered later (Chapter 6).

Females do not fully disappear from catches in the winter months, but low numbers occur during this period. Thomas & Figueiredo (1965) showed that immature sized females show no seasonal variation in abundance, indicating that females caught in the winter were much smaller than those caught in the summer months. Therefore it could be hypothesised that immature females do not have the same extended in-burrow periods as mature females do. This needs to be controlled for in the collection of females.

1.5 Seasonal differences

Food for the benthos descends through the water column (Stephens *et al.*, 1967) and therefore any reduction of food supply and thus nutritional status will be driven mainly from above. Fluctuations in primary production could change the amount of particulate organic material sinking at different times of the year, for example there will be a large influx due to the spring bloom. However, this would be affected by sinking time and the extent to which it is consumed as it falls by zooplankton or other pelagic animals. Stephens *et al.* (1967) showed that in the winter there was a reduction of total sedimentation as well as a reduction of organic carbon and nitrogen. There can be other seasonal fluctuations due to fishing effort and discarding rate. The introduction of carrion to the system could also create a large effect on nutritional status for a short time (Watts *et al.*, 2011).

In this thesis the extent to which such seasonal factors affect the nutritional status of both male and female *N. norvegicus* has been tested over one year (2009).

1.6 Site differences

Although no evidence exists for a significant genetic difference in populations of *N. norvegicus* throughout their entire range (Pompoulie *et al.*, 2011), various biochemical, life history and growth rate differences have been reported across their distribution range (Chapman & Bailey, 1987; Bell *et al.*, 2006).

The structure of the population of *N. norvegicus* in a particular area is driven by a number of different factors. Density is measured annually for commercially-exploited populations of *N. norvegicus* by ICES, using burrow count data from a sledge-mounted camera towed along the sea bed (ICES, 2011).

Chapman & Bailey (1987) showed that in certain areas there were high densities of small animals, while in other, sometimes adjacent, areas there were low densities of larger animals. *N. norvegicus* prefer sediments with a content of more than 40% silt and clay (Bell *et al.*, 2006). In Scotland there are high densities of burrows in coarse mud and lower densities of burrows in finer sediment (Tuck *et al.*, 1997b; Chapman & Bailey, 1987; Tuck *et al.*, 1999; Bell *et al.*, 2006; Campbell *et al.*, 2009). The density of *N. norvegicus* on Scottish grounds is driven by the dispersal of their planktonic larval life stages, since migration of adult *N. norvegicus* may never reach more than *ca* 100 metres from the burrow. Chapman & Bailey (1987) showed that the local hydrology determines the settling of the sediment, with high hydrographic energy areas laying down coarser sediments and low hydrographic energy areas laying down finer sediments. Chapman & Bailey (1987) suggest that the low energy areas are unsuitable for the movement of planktonic larvae. Therefore the coarse sediment and the larval *N. norvegicus* will settle in the same areas, and thus determine the high density (Tuck *et al.*, 1997b, c). The inverse is, however, the case in the Irish Sea (Tully & Hillis, 1995) and Mediterranean Sea (Maynou & Sarda, 1997), with higher densities on finer mud and lower densities on coarse mud (Bell *et al.*, 2006) as the finer sediment and *N. norvegicus* larvae are driven down to the deeper areas together. Afonso-Dias & Bailey (1998) and Campbell *et al.* (2009) showed that higher densities are only able to be supported if the substrate is sufficiently coherent to allow robust burrow systems to be excavated. They thus proposed that the relationship between *N. norvegicus*

burrow density and sediment particle size was dome shaped, and that sediments with medium particle sizes would be the best for constructing burrows.

Chapman & Bailey (1987) also hypothesised that the growth rates of *N. norvegicus* in these high density areas would be curtailed for three reasons. Firstly in high density areas, competition for food may limit scope for growth; secondly, the low silt and clay content means that there is low organic carbon content, which Chapman & Bailey (1987) attributed to the 'impoverished fauna' in the Sound of Jura (a high density area); and finally, the more aggressive social behaviour in high density areas could drive up the metabolic rate and thus energy requirements. Tuck *et al.* (1997b) also showed that the growth was negatively correlated to burrow density, and as a result suggested that higher densities will lead to food limitation. Parslow-Williams (1998) extended this work and showed that the nutritional status of animals in the areas of high densities, such as around Alisa Craig, are low when compared to areas around the island of Little Cumbrae.

Therefore differences in feeding could be due to natural differences in population density, habitat, or they could result from differences in the diversity of species available as prey items. Also, feeding could be affected by various anthropogenic factors that impact on ecosystem function, examples of which in the marine environment are pollution and fishing.

1.7 Sites within this study

The two sites which have been the focus of the present study are the Clyde Sea Area (sometimes considered synonymous with the Firth of Clyde), referred to hereafter in this thesis as the 'CSA', and the North Minch, hereafter referred to as the 'NM'. Both sites are within the ICES area VIa, with the stock of *N. norvegicus* in the NM forming the ICES Functional Unit 11 and that in the CSA and the Sound of Jura together forming Functional Unit 13 (ICES, 2011). These areas are shown in Figure 1.5. Both areas have commercially exploited populations of *N. norvegicus* and thus are regulated through the European Union (EU) Common Fisheries Policy.

The two sites have similar soft muddy sediments which are very suitable for *N. norvegicus* burrow systems. The area studied in the NM has a mean depth of 118.94 m ± 1.46, which is deeper than the area studied in the CSA, which has a mean depth of 82.61 m ± 1.12.

As mentioned above, the burrow density is linked to the growth rate and nutritional status of *N. norvegicus* (Tuck *et al.*, 1997 b, c and Parslow-Williams, 1998). As measured in 2009, the mean burrow density In the CSA was 0.85 m^{-2} compared with 0.55 m^{-2} in the NM (ICES, 2011). The findings of Chapman & Bailey (1987), Tuck *et al.* (1997b, c) and Parslow-Williams (1998) suggest that the *N. norvegicus* in the CSA may have a lower nutritional status than those in the NM, but this has now been tested directly in the present investigation.

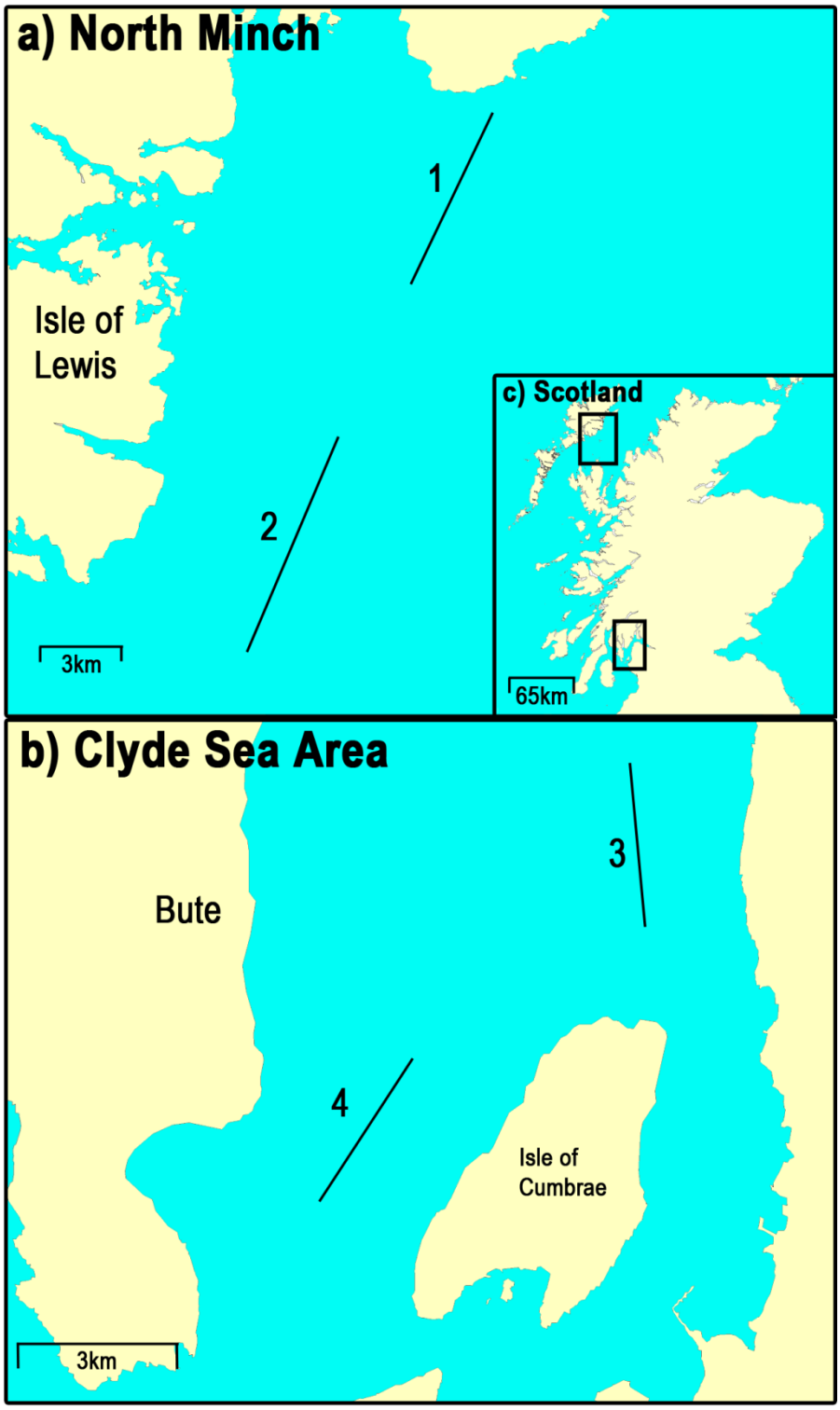

Figure 1.5 Sites sampled throughout this project. (a) The North Minch (NM), on MFV Comrade III sailing from Stornoway: Transect 1- East 58˚08.57'N 6˚09.13'W to 8˚05.34'N 6˚06.71'W; Transect 2- South 58˚02.71'N 6˚15.24'W to 7˚57.19'N 6˚15.74'W (b) The Clyde Sea Area (CSA) on RV Aora and RV Aplysia sailing from the University Marine Biological Marine Station Millport, Isle of Cumbrae: Transect 3- North 55˚51.35'N 4˚54.42'W to 55˚48.97'N 4˚54.05'W; Transect 4- West 55˚47.79'N 4˚58.33'W to 55˚44.88'N 4˚59.36'W.

1.8 Overview of thesis

The following six data chapters report studies aimed at answering some of the questions raised above. These studies fall into two groups, firstly those addressing the nutritional status of *N. norvegicus* (Section 1: Chapters 2-5), and secondly those relating to the trophic dynamics of *N. norvegicus* (Section 2: Chapters 6-7).

In order to establish how *N. norvegicus* can maintain themselves over a sustained period of starvation, a series of laboratory trials was carried out, firstly using males and then females, to produce a time series of how this species mitigates the stress of starvation (Chapters 2 and 3). Specifically, the timescales involved in the two phases of the starvation response, namely metabolic depression (Phase 1) and utilisation of fuel reserves (Phase 2) were studied. This integrated approach provides a greater possibility of understanding the animal's coping mechanisms to the stress of starvation, compared with the individual methods used previously. Chapter 4 attempts to develop biomarkers from the physiological responses to starvation which will help assess degree of starvation in field-sampled *N. norvegicus* of unknown nutritional status.

The seasonal variation in feeding behaviour of *N. norvegicus* within the two populations off the west coast of Scotland already mentioned were then studied (Chapter 5). Links to both the seasonal changes in the behaviour of females, and to food availability were investigated. This comparison between sites also tested the burrow density hypothesis to see if *N. norvegicus* at one site have a lower nutritional status than those at the other. The different seasonality, life history stages, gonad development and brooding periods were all assessed with regard to nutritional status. Using starvation measures from the previous section alongside ecological data from field samples obtained monthly or bimonthly from two sites (CSA and NM), a picture of nutritional status and how it changes over temporal and spatial scales has been developed.

Finally, an assessment has been made of how site variations affect the trophic dynamics and feeding preferences of *N. norvegicus* (Chapters 6 and 7). This was achieved by determining the trophic level of *N. norvegicus* at each site in

relation also to season and sex (Chapter 6). In addition, the compositions of the diets consumed by individuals at the two sites throughout the year were determined by fatty acid analysis (Chapter 7).

The final chapter of this thesis (Chapter 8) attempts to synthesise the results obtained to fully answer the questions raised in this Introduction, to consider the implications of the findings and also to broaden the application of this study to other fields of research.

2 Nutritional status of male *Nephrops norvegicus* under starvation conditions

2.1 Introduction

Many marine benthic animals have evolved to survive by employing a range of predatory and scavenging feeding behaviours. However, food availability can be sporadic in benthic ecosystems, with periods of time when food is not available. In response, many crustaceans, including *Nephrops norvegicus*, are nevertheless known to be able to survive for extended periods of time without food (Baden *et al.*, 1994 deprived *N. norvegicus* of food for 7 months), triggering a starvation response in the animal.

Therefore, in order to assess if the animal is starving in the wild it is firstly necessary to establish the starvation response of the animal and which mitigating processes they employ to survive this period. The two phases of starvation (namely metabolic depression and reserve utilisation) as described in Chapter 1 will be considered.

2.1.1 Measuring the two phases of starvation

2.1.1.1 Metabolic depression - Phase 1

N. norvegicus can regulate their metabolic rate in order to reduce their energy needs (Parslow-Williams *et al.*, 2002). This could contribute to their survival for extended periods without food. The most direct way to record this would be by measuring respiration, but this is not appropriate for assessing large numbers of specimens in field samples (animals are often obtained either moribund or dead), and therefore a proxy needs to be identified.

Dall (1981) suggested that metabolic rate decreases as the nutritional status reduces, which was demonstrated by Parslow-Williams (1998) who showed that O_2 consumption significantly decreased in starved *N. norvegicus* after 8 weeks without food. It has been found that the amount of haemocyanin (the O_2 carrier protein) decreases in the haemolymph in conjunction with such reductions in

metabolic rate (reviewed by Depledge & Bjerregaard, 1989). Since each haemocyanin molecule contains an active site of two copper atoms, an indirect way of estimating the amount of haemocyanin is to measure the concentration of copper in the haemolymph (Taylor & Anstiss, 1999). This indirect measure can also establish the fate of the copper atoms released when the haemocyanin molecules are broken down and was used by Spoek (1974). Taylor & Anstiss (1999) suggested that the hepatopancreas is the principal site for the accumulation of copper released in this way. Therefore when copper decreases in the haemolymph, it would be expected to increase in the hepatopancreas. Simultaneous measurements of copper concentration made on these two tissues could establish if this is the case.

2.1.1.2 Reserve utilisation phase - Phase 2

Metabolic reserves within the body of *N. norvegicus*, such as lipids and carbohydrates, could be utilised to provide energy when the animals are starving. Indeed, lipids contained within the cells of the hepatopancreas have been studied as potential indicators of starvation in decapod crustaceans. Barclay *et al.* (1983) showed that the lipid content of the hepatopancreas of the prawn *Penaeus esculentus* decreased after 7 days of starvation (in relation to a fed control). The hepatopancreas in *N. norvegicus* accounts for an average of 6% of the whole body weight (own data) and in some cases can be much higher. Therefore this store of lipids (around 30% of the hepatopancreas by weight) might be a source of exploitable internal reserves when food is absent. However, Dall (1981) found that after 18 days without food there was no decrease in the hepatopancreatic lipids of *N. norvegicus*, and he suggested that the metabolic rate could have decreased sufficiently to ensure that lipids were not utilised within this period of time. A change of fatty acid composition, he suggests, could nevertheless have occurred over this period. Furthermore, Dall (1981) did not investigate the starvation response beyond 18 days. Therefore it cannot be excluded that there is a decrease in hepatopancreatic lipids after this time, when other internal reserves have been depleted and the ecological factors mentioned in Chapter 1, such as females remaining in burrows, exert their effects. Glycogen, the stored form of carbohydrate, is a more readily available form of energy for animals. Glycogen is converted into glucose via glycolysis.

Stores of glycogen form when excess glucose and ATP is available through the process of glycogenesis. Under the stress of starvation, glycolysis is greater than glycogenesis and thus glycogen is depleted. Baden *et al.* (1994) showed that this occurs in both the tail and the hepatopancreas of *N. norvegicus* during starvation.

The protein in the tail muscle of *N. norvegicus* has been found not to decrease significantly with starvation throughout a 12-week period (Parslow-Williams, 1998) nor during 6 months (Mente *et al.*, 2011). Protein, in the form of haemocyanin within the haemolymph, when decreased in conjunction with a reduced metabolic rate as suggested in Taylor & Anstiss (1999), could also be used as a source of energy.

Rather than measuring the direct utilisation of reserves, the end state of the animal, as reflected in weight change, the water content and the chemical composition of various tissues could all be used to indicate the starvation response. More specifically, the ratio of carapace length cubed to weight change indicates weight in proportion to the size of the animal, and thus any loss in weight would increase this ratio. A change in weight of the hepatopancreas as a proportion of overall body weight, as expressed by the hepatosomatic index (HSI), can serve as a condition index (Jones & Obst, 2000). The water content of the tissues reflects the concurrent consumption of resources in both the tail and the hepatopancreas (Barclay *et al.*, 1983).

Stable Isotope Analysis (SIA) has also been used to indicate starvation in many animals such as the flatworm *Arthurdendyus triangulatus*, and a collembolan arthropod species (Haubert *et al.*, 2005), the Nile Tilapia *Oreochromis niloticus* (Gaye-Siessegger *et al.*, 2007), reptiles (McCue & Pollock, 2008) and sea birds (Williams *et al.*, 2007), including penguins *Aptenodytes patagonicus* (Boag *et al.*, 2006; Cherel *et al.*, 2005). Nitrogen isotope fractionation (as defined later in Chapter 6) occurs between diet and tissue due to a preferential excretion of ^{14}N, which results in a bioaccumulation of ^{15}N from the diet. Normal trophic shifts therefore produce increases of $\delta^{15}N$. When the animal is starved, tissue breakdown replaces feeding, and therefore $\delta^{15}N$ is expected to increase (Figure 2.1).

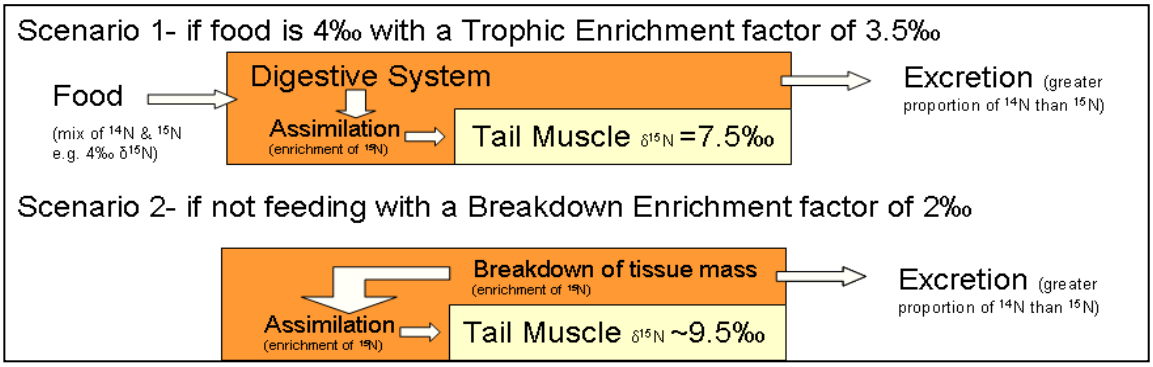

Figure 2.1 Two scenarios to explain how starvation might affect the stable isotope ratios of an animal. Scenario 1 - feeding. All organic material (dead or alive) has a $\delta^{15}N$ ratio. If this material is eaten the animal will digest the nitrogeneous compounds and its excretions will have a greater proportion of ^{14}N to ^{15}N. As a consequence, the nitrogen assimilated into the tissues will have a greater proportion of ^{15}N to ^{14}N, and accordingly, the ^{15}N in the tail muscle level will be higher than in the food. Scenario 2 – starvation. If the tail muscle protein is broken down as an energy source then a greater proportion of ^{14}N will still be excreted. Meaning that the tail muscle would increase in $\delta^{15}N$ compared with scenario 1 (as it is 'feeding' on itself). NB: trophic and breakdown enrichment values are hypothetical for illustrative purposes only.

2.1.2 Aims of the Chapter

Individual measures of starvation have been used in past studies. However, such measures have rarely been used in combination to make the analysis more robust. This chapter is therefore concerned with measures that could be used subsequently (see Chapter 4) to create a model to represent robust and sensitive biomarkers for starvation in field-caught animals. For this purpose, a series of controlled starvation trials was carried out in an aquarium. Only male *N. norvegicus* were used in this study. Starvation measures are assessed in females in Chapter 3.

In both male (this chapter) and female (the following chapter) trials a range of different measures of starvation were made on both fed and unfed groups of animals. With the results obtained, each measure was then assessed as a potential biomarker according to three criteria.

1. There is a clear difference between fed and unfed animals
2. This difference remains beyond a certain time point
3. It is insensitive to short term stresses caused by the method of collection (e.g. trawl capture).

2.2 Materials and Methods

2.2.1 Starvation study

This aquarium trial was carried out with male *N. norvegicus*.

The animals were caught by otter trawl on 27 Jan 2010 from the Clyde Sea Area-north transect (Figure 1.5), UK (55˚51.351′ N 4˚54.424′ W to 55˚48.979′ N 4˚54.055′ W) by the RV Aora (UMBSM). Males with a mean carapace length (±SD) of 35.1 ± 5.88 mm were selected from the landed catch. These were then returned alive to the University of Glasgow on sea ice in sealed containers, and were held in an aquarium supplied with re-circulating filtered seawater for 2 weeks (with a 12h:12h light: dark photoperiod,11.7°C ± 0.1 for the full study) to allow them to recover from the stresses of capture and transport. Tanks were checked daily and any dead animals were removed.

After the 2 week acclimatisation period the animals were moved temporarily into large tubs while the experimental tanks were cleaned, and were then randomly assigned to one of 14 tanks and one of six sections in each of these tanks (held at the same conditions as mentioned above). All animals were fed on *ca* 1 g squid mantle (the 'standard food ration') three times a week for two weeks, until the trial started on 02 March 2010, after which time feeding continued with the 'standard food ration' in half of the tanks (fed group), while in the remaining tanks feeding was discontinued (unfed group). One lobster from 12 tanks was removed every 4 weeks (weeks 0, 4, 8, 12, 16, 20), and put on ice for 10 minutes. 61 out of 84 animals were selected throughout the trial, mortality was low in the other animals. Carapace length and weight with claws were measured, *ca* 1 ml of haemolymph was removed from the base of the fifth pereiopod and the animal was reweighed without chelipeds. This species will readily autotomise its chelipeds under stressful conditions (including trawling and handling), so this approach eliminates variation in the final CL:Wt and HSI values. Chelipeds have been removed in a number of previous similar studies, including that of Sarda & Valladares (1990).

The hepatopancreas was then removed from the cephalothorax (noting the colour and weight), and the entire muscle was removed from the tail, prior to these tissues being flash frozen in liquid nitrogen and then stored at -80°C prior to further analysis.

2.2.2 Measurements

Tissue samples were analysed after the end of the trial in a random order, to minimise the possibility of errors introducing bias to the measurements of samples from different periods of starvation. Samples were randomised by assigning a random number to each individual lobster in Microsoft Excel® and sorting them into numerical order: thus there was little chance of week 20 animals being measured differently from week 0 animals. When it was not possible to measure all the animals in one session (as in the case of lipids), each sequence of measurements was made on a selection of all starvation periods.

2.2.2.1 Water content

Prior to any biochemical measures being made on any of the collected tissue samples, the whole hepatopancreas together with a proportion of tail muscle was freeze-dried for 7 days, noting the initial wet weights and final dry weights in order to allow their water content to be calculated.

Water content of tissue, WC (%), was calculated as

$$\mathrm{WC} = 100\left(1 - \frac{DW_t}{WW_t}\right) \qquad \text{Equation 2.1}$$

Where;
DW_t is the dry weight of the tissue
WW_t is the wet weight of the tissue.

2.2.2.2 Copper determination

The copper determination protocol was modified from Mohamad (2008) with advice from Perkin Elmer Ltd. using a modified digester method. Approximately

100 mg of dry hepatopancreas or 1 g of frozen haemolymph was digested in 8 ml of concentrated nitric acid (HNO_3) and heated at 95˚C for 2 h in plastic 'Digi prep' tubes on a 48-well sample digester (SPB 50-48 & SPB Digital controller, Perkin Elmer Ltd., Cambridge, UK). Samples were allowed to cool for 10 min and 3 ml of hydrogen peroxide (H_2O_2) was added to complete the digestion. Samples were left to cool overnight, prior to being made up to 10 ml with distilled water. Standards of copper (Fluka, Sigma Aldrich) were prepared with concentrations of 10 ppm, 5 ppm, 2.5 ppm and 1.25 ppm. Distilled water was used as a blank to represent 0 ppm. Copper concentration was determined by means of Atomic Absorption Spectrometry (AA Analyst400, Perkin Elmer Ltd, Cambridge, UK). Reference samples of TORT-2 (National Research Council Canada Certified reference material of lobster hepatopancreas) were also analysed with each digest session. Copper was displayed as micrograms of copper per gram wet tissue weight ($\mu g.g^{-1}$).

2.2.2.3 Glycogen determination

Glycogen was determined via the protocol set out in Albalat *et al.* (2010) Approximately 90 mg of frozen tail muscle and 70 mg of dry hepatopancreas was weighed into separate labelled 1.5 ml microcentrifuge tubes to which 400 µl of 30% (w/v) potassium hydroxide was added. This was boiled for 45 min for tail muscle and 3 h for hepatopancreas until the sample was orange in colour with no solid particles left. Samples were left to cool for 10 min then 500 µl of absolute ethanol was added, mixed and left to stand on ice for 2 h. Samples were then centrifuged for 15 min at 5˚C at 15,000 ***g***. The supernatant was removed and 500 µl of distilled water added to the remaining pellet. These samples were frozen at -20°C until required.

Samples were allowed to defrost at room temperature and were sonicated to ensure that the pellet was dissolved. 50 µl of sample was added to another 1.5 ml microcentrifuge tube followed by 1 ml of anthrone reagent (72 ml concentrated sulphuric acid gradually added to 28 ml distilled water held in ice, followed by 50 mg of powdered anthrone). The samples were boiled for 10 min and cooled on ice for 10 min. These samples were loaded on to flat-bottom 96-well plates and absorbance at 620 nm was recorded on a spectrophotometer (Thermo Scientific Multiskan spectrum 1500, Thermo Scientific inc. Waltham,

USA), along with standards of 1 mM, 2.5 mM, 5 mM and 10 mM of glucose to which anthrone reagent had been added. Glycogen was displayed as wet weight in the units $mg.g^{-1}$.

2.2.2.4 Protein determination

Protein determination was carried out by a method described in Parslow-Williams (1998), but using the more moden BCA reagent rather than Lowry. Approximately 10 mg of a sample of dry tail muscle was placed into a 25 ml plastic centrifuge tube. 10 ml 0.1N NaOH was added and vortexed. This was left to sit over night. The sample was then centrigfuged at 6000 ***g*** for 20 mins. 0.5 ml of the supernatant was then passed to a labelled 1.5 ml microcentrifuge tube to which 1ml aliquots of 10% trichloroacetic acid was added, vortexed and left to stand overnight. This tube was then centrifuged at 13,000 ***g*** for 20 mins. The supernatants were then discarded and a further 0.5 ml of 0.1N NAOH was added. A BCA working reagent was made up by adding 50 parts bicinchoninic acid (Sigma B9643) to 1 part copper (II) sulphate pentahydrate 4% solution (Sigma C2284). A standard curve of Bovine serum albumin standard was prepared 1 $mg.ml^{-1}$, 0.8 $mg.ml^{-1}$, 0.6 $mg.ml^{-1}$, 0.4 $mg.ml^{-1}$, 0.2 $mg.ml^{-1}$ with NaOH as the buffer, a blank of NaOH was used to represent 0 $mg.ml^{-1}$. Samples and standards were then added in duplicate to a 96-well plate along with 200 µl BCA working reagent (8:1) into each well. The plate was then incubated 37˚C for 30 minutes and measured at an absorbance of 562 nm. Protein was displayed as percentage dry weight.

2.2.2.5 Lipid determination

Lipid determination was carried out via the Folch Method (Folch *et al.*, 1957). Approximately 100 mg of a sample of dry hepatopancreas or the remainder tail muscle sample, was placed in a 50 ml borosilicate glass 'quickfit' tube, a volume of 20 ml chloroform/methanol mixture (C:M 2:1 v:v) was added, and the tube was placed on ice. These samples were then homogenised, with the probe being washed in C:M (2:1) between samples. Stoppers were added and the samples were left to stand for 1 h. A volume of 5 ml of 0.88% (w/v) potassium chloride (KCl) was added to each homogenised sample, which was then vortexed and left to stand for 5 min. The samples were centrifuged at 400 ***g*** for 5 min, and the

uppermost layer was removed by aspiration. The bottom layer was transferred to 20 ml, pre-weighed, 'quickfit' tubes through prewashed (with C:M 2:1) Whatman No.1 filter paper. The solvent was evaporated to dryness at 25˚C under a stream of oxygen-free nitrogen (OFN), and the extracts within the tubes were desiccated overnight. The tubes with the dry desiccated lipid were reweighed and the percentage of lipid determined. Samples were then re-dissolved in C:M (2:1) and 0.01% (w/v) butylated hydroxytoluene (BHT) at a concentration of 10 $mg.ml^{-1}$. The dissolved lipids were stored in small glass tubes at -20˚C, with oxygen replaced by nitrogen, to ensure oxidation of the lipid did not take place. Lipids were displayed as percentage wet weight.

2.2.2.6 Equations for indices and ratios

The carapace length: weight ratio, CL:W, was calculated as

$$\mathrm{CL:W_b} = 100\frac{CL^3}{W_b} \qquad \text{Equation 2.2}$$

Where;
CL is the carapace length (mm)
W_b is wet weight of the body minus chelipeds (g).

The hepatosomatic index, (HSI), was calculated as

$$\mathrm{HSI} = 100\frac{W_h}{W_b} \qquad \text{Equation 2.3}$$

Where;
W_h is the wet weight of the hepatopancreas
W_b is the wet weight of the body without chelipeds.

2.2.2.7 Stable Isotope Analysis (SIA)

Stable Isotope Analyses were carried out on untreated freeze-dried tail muscle tissue and hepatopancreas samples (0.5–0.7 mg weighed aliquots to an accuracy of 4 decimal places in tin capsules on a Mettler MX5 balance (Mettler-Toledo international Inc., Columbus, USA)). These analyses were conducted at the East

Kilbride node of the NERC Life Sciences Mass Spectrometry Facility hosted by Scottish Universities Environmental Research Centre (SUERC) using a continuous flow isotope ratio mass spectrometer (Thermo Fisher Delta XP Plus, Thermo Scientific Bremen, Germany) and a Costech ECS 4010 elemental analyser (Costech Analytical Technologies inc., Milan, Italy). Drift and linearity were corrected by the use of laboratory standards of gelatine (reproducibility [SD] of around 0.18‰ for $\delta^{15}N$ and 0.10‰ for $\delta^{13}C$) and alanine, which are routinely checked against international isotope standards from the International Atomic Energy Agency (IAEA) and National Institute of Standards and Technology (NIST).

2.2.3 Statistical analysis

2.2.3.1 General Linear Models (GLMs)

Statistical analyses were carried out for each measure by a (GLM) treating fed and unfed animals as separate experiments. The response variable was the biochemical measure and the explanatory variable was week of the treatment (as a categorical factor). The residuals were assessed visually for normality. When the overall test indicated a significant difference ($P<0.05$) among weeks, multiple comparisons were made with post hoc Tukey comparison test ($P<0.05$). Significant P values will be displayed in bold when appearing in tables. Error values represent one standard error (± SE).

Total number of observations in each table and figure where appropriate are reported as n=# or (when statistical output is present in tables) n=total df + 1, a breakdown of numbers of observations in each factor is displayed within the appendices.

2.3 Results

A total of 72 male *N. norvegicus* were sampled throughout the trial (6 animals for each treatment at 6 time periods). The GLM output, showing the significance of the difference among weeks from week 0 to week 20 for both fed and unfed groups, along with the means and standard error values, are shown in Table 2.1. The following sections explain the results of this table and how different

measurements were affected during the starvation trial. All analyses were conducted on the basis of assessing the measure as a difference among week points.

The mean sizes (CL) of the animals within the groups, sampled at each 4 week time point, can be seen in Figure 2.2. Size did not vary significantly in the unfed ($F_{5,27}$=0.97, P=0.457) or fed ($F_{5,31}$=1.71, P=0.168) groupings among the week sampling points, indicating that there was no size bias in the sampling protocol.

2.3.1 Effect of food removal on *N. norvegicus*

2.3.1.1 Metabolic depression - Phase 1

Copper

The mean values (± SE) of the copper concentration of the a) hepatopancreas and b) haemolymph in male *N. norvegicus*, between fed and unfed animals from each 4 week experimental period, are shown in Figure 2.3.

The copper concentration in the hepatopancreas of the animals in the unfed group increased significantly from 153.53 ± 42.17 $\mu g.g^{-1}$ at week 0 to 456.50 ± 152.77 $\mu g.g^{-1}$ in animals selected at week 16 ($F_{5,23}$=3.77, P=0.016). The mean copper concentration then decreased significantly to 169.08 ± 97.83 $\mu g.g^{-1}$ in animals selected at week 20 (Tukey, P<0.05). The copper concentration in the fed animals did not vary significantly throughout the trial ($F_{5,30}$=0.91, P=0.490).

The copper concentration in the haemolymph in the unfed group decreased significantly from 84.54 ± 23.71$\mu g.g^{-1}$ at week 0 to 25.16 ± 5.48 $\mu g.g^{-1}$ at week 12 ($F_{5,26}$=4.05, P=0.010). The haemolymph copper concentration then remained constant at 33.37 ± 11.02 $\mu g.g^{-1}$ and 34.73 ± 2.70 $\mu g.g^{-1}$ in the animals selected in weeks 16 and 20, respectively. There was no significant variation among weeks in the haemolymph copper concentration of fed animals ($F_{5,28}$=0.82, P=0.548).

2.3.1.2 Reserve utilisation- Phase 2

Glycogen

The mean values (± SE) of the glycogen concentration of the a) hepatopancreas and b) tail muscle in male *N. norvegicus*, in fed and unfed animals from each 4 week experimental period, are shown in Figure 2.4.

The glycogen content of the hepatopancreas in unfed individuals varied significantly among weeks ($F_{5,17}$=3.30, P=0.042). In week 0 the mean glycogen concentration of the hepatopancreas was 2.21 ± 1.08 $mg.g^{-1}$. The animals sampled after 4 weeks had a significantly elevated mean value of 20.01 ± 6.91 $mg.g^{-1}$. The glycogen concentration in the hepatopancreas of fed animals did not vary significantly among weeks ($F_{5,29}$=1.90, P=0.121); however, it was consistently higher than that of unfed individuals. The glycogen content of the tail muscle did not vary significantly among weeks in either fed ($F_{5,29}$=1.90, P=0.132) or unfed animals ($F_{5,27}$=1.11, P=0.385).

Protein

The mean values (± SE) of the protein content of the tail muscle in male *N. norvegicus*, between fed and unfed animals from each 4 week experimental period, are shown in Figure 2.5.

The protein content of the tail muscle in unfed individuals varied significantly among weeks ($F_{4,22}$=3.74, P=0.022); this was due to animals at week 8 (23.71 ± 0.94%) being significantly lower than animals at week 4 (32.75 ± 1.28%) and animals at week 16 (32.38 ± 0.78%). Animals sampled at week 8 were, however, not significantly different from animals at week 0 (29.67 ± 0.76%) or at week 12 (30.48 ± 2.89%) (Tukey, P<0.05). The protein content of the tail muscle was not significantly different among the week periods in fed individuals ($F_{4,21}$=1.88, P=0.160).

Lipid

The mean values (± SE) of the lipid content of the hepatopancreas in male *N. norvegicus*, between fed and unfed animals from each 4 week experimental period, are shown in Figure 2.6.

The lipid content of the hepatopancreas decreased significantly during starvation ($F_{5,23}$=4.60, P=0.007). The mean lipid content of the hepatopancreas in animals sampled at week 0 was 15.22 ± 2.78%. There was then a significant reduction in hepatopancreas lipid content in unfed lobsters between week 4, with a mean lipid content of 17.00 ± 2.58%, and week 20, with a mean lipid content of 2.58 ± 1.32% (Tukey, P<0.05). In contrast, there was no significant difference in hepatopancreas lipid content of fed animals among weeks ($F_{5,26}$=0.86, P=0.523).

Carapace length: weight ratio

The mean values (± SE) of the carapace length: weight ratio of male *N. norvegicus*, between fed and unfed animals from each 4 week experimental period, are shown in Figure 2.7.

The CL: weight ratio in male *N. norvegicus* that were unfed did not vary significantly during the whole period of food deprivation ($F_{5,27}$=0.72, P=0.618). Male *N. norvegicus* that were maintained on the standard ration also did not vary significantly throughout the trial ($F_{5,31}$=1.12, P=0.377).

Hepatosomatic index (HSI)

The mean values (± SE) of the hepatosomatic index in male *N. norvegicus*, between fed and unfed animals from each 4 week experimental period, are shown in Figure 2.8.

The HSI declined in unfed animals with time ($F_{5,28}$=12.10, P<0.001). The mean HSI in unfed animals was 6.34 ± 0.38% at week 0 and was significantly reduced to 4.03 ± 0.44% by week 8, to 2.56 ± 0.23% in animals selected after week 12, to 3.06 ± 0.35% in animals selected at week 16 and to 2.19 ± 0.50% in animals selected after week 20.

Water content of tissues

The mean values (± SE) of the water content of the a) hepatopancreas and b) tail muscle in male *N. norvegicus*, between fed and unfed animals from each 4 week experimental period, are shown in Figure 2.9.

The water content of the hepatopancreas increased significantly in unfed animals over time ($F_{5,28}$=7.97, P<0.001). Mean water content was significantly

higher at week 12 (77.28 ± 1.32%), week 16 (75.62 ± 2.39%) and week 20 (80.21 ± 1.56%) than at week 4 (63.13 ± 2.68%) (Tukey, $P<0.05$). The water content of the tail muscle did not differ significantly with the length of trial period in unfed ($F_{5,28}=1.36$, $P=0.276$) or fed ($F_{5,31}=0.57$, $P=0.725$) animals. Hepatopancreas water and lipid contents are very strongly correlated with each other (Pearson's $r=-0.966$ $P<0.001$): as lipids decrease water content increases.

Stable isotopes

The mean values (± SE) of the stable isotopes of a) $\delta^{15}N$ and b) $\delta^{13}C$ in the tail muscle of male *N. norvegicus*, between fed and unfed animals from each 4 week experimental period, are shown in Figure 2.10

The tail muscle $\delta^{15}N$ of unfed animals did not differ significantly between week 0 and week 20 ($F_{5,28}=0.06$, $P=0.997$). However, fed animals did vary significantly among weeks ($F_{5,31}=2.98$, $P=0.029$). At week 0 the mean $\delta^{15}N$ value of the tail muscle was 13.22 ± 0.23‰, which was lower than in animals selected at week 16 (13.74 ± 0.17‰) and at week 20 (13.75 ± 0.24‰).

The $\delta^{13}C$ values of the tail muscles of the unfed individuals did not vary significantly in unfed ($F_{5,28}=1.24$, $P=0.322$) or fed animals ($F_{5,31}=1.65$, $P=0.181$) among weeks.

The mean values (± SE) of the C:N ratio of the tail muscle in male *N. norvegicus*, between fed and unfed animals from each 4 week experimental period, are shown in Figure 2.11.

The tail muscle C:N ratio in unfed animals reduced significantly throughout the trial ($F_{5,27}=7.50$, $P<0.001$). The animals at the start of the trial had a mean tail muscle C:N ratio of 3.16 ± 0.04 which was significantly higher than the C:N ratio of animals selected at week 12 (3.04 ± 0.02), at week 16 (3.01 ± 0.02), and at week 20 (2.99 ± 0.03) (Tukey, $P<0.05$). One animal was removed from the analysis from week 8 as it had an abnormally high C:N ratio for tail muscle (3.77) due to the animal moulting a week previously.

The mean tail muscle lipid content in 6 unfed animals sampled week 16 and 20 (0.70 ± 0.05%) was significantly lower than the mean tail muscle lipid content of

fed individuals (0.96 ± 0.06%) ($F_{1,11}$=12.39, P=0.006). Figure 2.12 shows that the six highest and six lowest C:N ratio values correlate with the lipid content of the tail muscle. Thus the change in C:N ratio could be directly related to the reduction in tail lipids (Pearson's r=0.610, P=0.035).

The mean values (± SE) of the stable isotopes of a) δ^{15}N and b) δ^{13}C in the hepatopancreas of male *N. norvegicus*, between fed and unfed animals from each 4 week experimental period, are shown in Figure 2.13.

There was no significant increase of hepatopancreas values in unfed animals for δ^{15}N ($F_{5,13}$=2.66, P=0.106) or δ^{13}C ($F_{5,13}$=2.59, P=0.112). However, the δ^{15}N value for the hepatopancreas was significantly different in fed animals ($F_{5,19}$=3.46, P=0.030).

The mean values (± SE) of the C:N ratio of the hepatopancreas in male *N. norvegicus*, between fed and unfed animals from each 4 week experimental period, are shown in Figure 2.14. The C:N ratios in the hepatopancreas of the unfed groups did decrease from the start, but this was not a significant reduction ($F_{5,13}$=1.60, P=0.264).

Table 2.1 GLM result comparing a) unfed animals b) fed animals among weeks. Separate post hoc Tukey tests to compare means between week (for unfed and fed) were carried out when significance was found (P<0.05). Week group means that do not share a letter are significantly different (P<0.05). initial n= 29 unfed, 32 fed.

a)	Among all unfed				Mean values ± SE, with results of unfed Tukey comparisons					
Measurement	**F**	**df**	**P**	**r^2 (%)**	**0**	**4**	**8**	**12**	**16**	**20**
Carapace length (mm)	0.97	5,27	0.457	18.07	38.95 ± 3.12	30.83 ±1.19	40.02 ± 3.65	37.42 ± 3.03	36.92 ± 2.59	37.93 ± 3.83
Hepatopancreas copper (µg.g^{-1} wet)	3.77	5,23	**0.016**	51.17	153.53 ± 42.17 **b**	287.51 ± 65.14 **ab**	294.12 ± 80.35 **ab**	423.37 ± 158.88 **a**	456.50 ± 152.77 **ab**	169.08 ± 97.83 **ab**
Haemolymph copper (µg.g^{-1} wet)	4.05	5,26	**0.010**	49.09	84.54 ± 23.71 **a**	65.60 ± 3.67 **ab**	48.27 ± 7.74 **ab**	25.16 ± 5.48 **b**	33.37 ± 11.02 **b**	34.73 ± 2.70 **ab**
Hepatopancreas glycogen (mg.g^{-1} wet)	3.30	5,17	**0.042**	57.90	2.21 ± 1.08 **b**	20.01 ± 6.91 **a**	4.31 ± 1.85 **ab**	3.67 ± 0.21 a**b**	1.70 ± 0.27 **ab**	2.74 **ab**
tail muscle glycogen (mg.g^{-1} wet)	1.11	5,27	0.385	20.11	2.31 ± 0.94	7.38 ± 5.05	3.73 ± 2.07	0.75 ± 1.06	1.74 ± 1.02	0.33 ± 0.17
tail muscle protein (% dry)	3.74	4,22	**0.022**	45.39	29.67 ± 0.76 **ab**	32.75 ± 1.29 **a**	23.71 ± 0.94 **b**	30.44 ± 2.88 **ab**	32.38 ± 0.78 **a**	-
Hepatopancreas lipid (% wet)	4.60	5,23	**0.007**	56.08	15.22 ± 2.78 **a b**	17.00 ± 2.58 **a**	11.05 ± 3.25 **ab**	5.63 ± 2.02 **ab**	6.75 ± 2.48 **ab**	2.58 ± 1.32 **b**
CL: weight Ratio	0.72	5,27	0.618	14.01	1296.76 ± 41.83	1348.21 ± 52.20	1426.56 ±104.25	1359.21 ± 45.26	1480.16 ± 93.05	1332.08 ±106.36
HSI (% wet)	12.10	5,28	**<0.001**	72.46	6.34 ± 0.38 **a**	4.53 ± 0.58 **ab**	4.03 ± 0.44 **bc**	2.56 ± 0.23 **bc**	3.06 ± 0.35 **c**	2.19 ± 0.50 **c**
Hepatopancreas water (%)	7.97	5,28	**<0.001**	63.39	68.05 ± 2.22 **bc**	63.13 ± 2.68 **c**	69.43 ± 2.93 **bc**	77.28 ± 1.32 **ab**	75.62 ± 2.39 **ab**	80.21 ±1.56 **a**
tail muscle water (%)	1.36	5,28	0.276	22.79	77.04 ±0.49	76.16 ± 0.45	77.00 ± 0.52	77.36 ± 0.46	77.19 ± 0.74	78.17 ± 0.49
tail muscle $\delta^{15}N$ (‰)	0.06	5,28	0.997	1.35	13.19 ± 0.07	13.11 ± 0.04	13.23 ± 0.30	13.22 ± 0.10	13.19 ± 0.15	13.22 ± 0.28
tail muscle $\delta^{13}C$ (‰)	1.24	5,28	0.322	21.08	-15.76 ± 0.16	-15.56 ± 0.11	-15.73 ± 0.27	-15.61 ± 0.14	-15.18 ± 0.18	-15.41 ± 0.27
tail muscle C: N ratio (wt)	7.50	5,27*	**<0.001**	63.02	3.16 ± 0.04 **a**	3.16 ± 0.02 **a**	3.20 ± 0.14 **ab**	3.04 ± 0.02 **b**	3.01 ± 0.02 **b**	2.99 ± 0.03 **b**
Hepatopancreas $\delta^{15}N$ (‰)	2.66	5,13	0.106	62.40	12.79 ± 0.16	12.22 ± 0.14	13.33 ± 0.25	13.57 ± 0.42	12.58 ± 0.65	12.95
Hepatopancreas $\delta^{13}C$ (‰)	2.59	5,13	0.112	61.78	-21.06 ± 0.64	-21.10 ± 0.24	-19.81 ± 1.23	-17.46 ± 0.18	-20.61 ± 0.59	-19.75
Hepatopancreas C: N (wt)	1.60	5,13	0.264	49.97	8.78 ± 1.39	7.73 ± 0.37	6.65 ± 1.88	3.83 ± 0.04	7.23 ± 0.45	5.47

*** REMOVED OUTLIER AT WEEK 8 WITH A VALUE OF 3.77 (RECENTLY MOULTED ANIMAL)**

Table 2.1 *cont.*

b)	Between all fed				Mean values ± SE, with results of fed Tukey comparisons					
	F	**df**	**P**	**r^2 (%)**	**0**	**4**	**8**	**12**	**16**	**20**
Carapace length (mm)	1.71	5,31	0.168	24.72	30.47 ± 1.62	33.50 ± 0.98	36.90 ± 1.96	36.03 ± 1.66	35.82 ± 2.10	37.76 ± 1.58
Hepatopancreas copper ($\mu g.g^{-1}$ wet)	0.91	5,30	0.490	15.40	292.61 ± 99.13	184.51 ± 54.47	211.06 ± 59.01	108.81 ± 39.06	140.48 ± 46.29	155.00 ± 37.66
Haemolymph copper ($\mu g.g^{-1}$ wet)	0.82	5,28	0.548	15.13	90.17 ± 12.70	68.08 ± 13.21	66.46 ± 18.04	67.99 ± 12.11	47.71 ± 10.57	71.68 ± 15.51
Hepatopancreas glycogen ($mg.g^{-1}$ wet)	1.98	5,27	0.121	31.09	18.85 ± 11.96	16.73 ± 0.50	15.62 ± 5.97	37.50 ± 5.98	36.11 ± 8.62	29.50 ± 5.87
tail muscle glycogen ($mg.g^{-1}$ wet)	1.9	5,29	0.132	28.32	3.10 ± 1.60	5.41 ± 1.79	6.15 ± 1.42	9.56 ± 1.77	5.47 ± 2.49	9.61 ± 0.59
tail muscle protein (% dry)	1.88	4,21	0.160	30.67	26.50 ± 3.55	28.98 ± 1.17	23.98 ± 1.64	30.33 ± 2.01	32.13 ± 2.01	-
Hepatopancreas lipid (% wet wt)	0.86	5,26	0.523	17.01	16.52 ± 1.43	15.82 ± 3.54	12.07 ± 2.97	18.03 ± 2.22	12.98 ± 0.92	11.79 ± 1.76
CL: weight ratio	1.12	5,31	0.377	17.66	1425.86 ± 49.55	1333.53 ± 42.40	1465.88 ± 70.82	1431.22 ± 41.09	1464.58 ± 57.00	1372.63 ± 22.81
HSI (% wet wt)	1.47	5,31	0.233	22.07	5.76 ± 0.42	5.69 ± 0.57	5.18 ± 0.35	5.07 ± 0.60	4.71 ± 0.44	6.34 ± 0.25
Hepatopancreas water (% wt)	0.73	5,31	0.616	12.25	63.93 ± 2.12	67.33 ± 3.50	69.55 ± 3.06	66.12 ± 2.24	67.76 ± 1.51	71.06 ± 1.91
tail muscle water (% wt)	0.57	5,31	0.725	9.82	75.88 ± 0.05	75.97 ± 0.79	76.74 ± 0.34	75.80 ± 0.55	75.68 ± 0.38	76.36 ± 0.60
tail muscle $\delta^{15}N$ (‰)	2.98	5,31	**0.029**	36.14	13.22 ± 0.23 **a**	13.56 ± 0.03 **a**	13.36 ± 0.12 **a**	13.16 ± 0.05 **a**	13.74 ± 0.17 **a**	13.75 ± 0.24 **a**
tail muscle $\delta^{13}C$ (‰)	1.65	5,31	0.181	24.14	-16.07 ± 0.10	-15.77 ± 0.13	-15.96 ± 0.14	-16.14 ± 0.15	-16.20 ± 0.13	-16.25 ± 0.16
tail muscle C: N ratio (wt)	0.84	5,31	0.536	13.85	3.18 ± 0.01	3.13 ± 0.02	3.15 ± 0.03	3.16 ± 0.02	3.15 ± 0.02	3.19 ± 0.03
Hepatopancreas $\delta^{15}N$ (‰)	3.46	5,19	**0.030**	55.30	12.19 ± 0.12 **a**	12.61 ± 0.17 **a**	13.02 ± 0.07 **a**	12.88 ± 0.13 **a**	12.68 ± 0.38 **a**	12.30 ± 0.10 **a**
Hepatopancreas $\delta^{13}C$ (‰)	1.06	5,19	0.424	27.39	-21.58 0.29	-21.64 ± 0.24	-21.65 ± 0.24	-22.07 ± 0.20	-21.29 ± 0.18	-21.33 ± 0.30
Hepatopancreas C: N (wt)	1.30	5,19	0.319	31.72	10.21 0.40	9.77 ± 1.18	10.89 ± 1.43	10.33 ± 0.94	8.03 ± 0.61	8.45 ± 0.93

Change in hepatopancreas composition

The composition of the hepatopancreas at each sample period in the trial is shown in Figure 2.15. It is expressed in two ways: firstly, (a) as a percentage of the hepatopancreas wet weight, which shows that the combined values of water and lipid stay relativity steady week-on-week; and secondly, (b) in relation to the size of the hepatopancreas as a percentage of the whole body weight. The

sum total of each constituent part equates to the mean HSI (percentage weight of hepatopancreas to whole body weight), and shows how the proportions of the constituents change throughout the trial.

The first graph shows that the percentage of lipids and water contained in the hepatopancreas accounts for *ca* 80% of the total weight of the hepatopancreas throughout the trial. Thus at week 0 the lipids and water combined accounted for 82.27 ± 0.36% of the total weight, in comparison to 80.13 ± 0.12% after week 4 and 82.79% ± 0.58 after week 20.

2.3.2 Moult effect

The individual values of the measured parameters in recently moulted animals were compared to the mean values for each sampled group at each time point (Table 2.2). This shows the effect that individuals in the pre or post-moult state had on the mean value for each group. Eight animals in this trial were found to be close to or have just completed ecdysis. One was in pre-ecdysis (identified through the presence of gastroliths), five were in post-ecdysis ('jelly' condition) and two were 'soft' (with uncertainty to whether they were in pre- or post-ecdysis since a 'paper-shell' condition can reflect calcium being withdrawn before moult as well as the early stages of subsequent redeposition). All the unfed moulting individuals had parameter values indicative of a higher nutritional status than the remainder of the group. This was indicated by a lower water content, a larger HSI and greater lipid content of the hepatopancreas, as well as a higher C:N ratio for two of the three moulted unfed individuals (one post moult animal had a C:N ratio of 3.77 as this was much greater all other C:N ratios it was removed from the analysis). However, the changes between week 4 and week 8 appear to be greater than the effect due to peri-moult conditions alone, and all values in moulted animals follow the trend of the data.

Table 2.2 Mean and standard error for each week - group of fed and unfed individuals for each parameter. Red numbers indicate animals which were soft when removed from the tank or had moulted during the trial. Explanation of each animal: (4a) ecdysis 1 week earlier; (4b) soft (unsure of pre/post-edysis); (8a) post-ecdysis 6 weeks earlier; (12c) premoult gastroliths; (16c) soft (unsure of pre/post-edysis); (20 c,d) ecdysis 2 weeks earlier; (20e) ecdysis 15 weeks earlier.

Water content of the hepatopancreas

	Not fed				Fed				
Week	**Mean**	**SE**	**a**	**b**	**Mean**	**SE**	**c**	**d**	**e**
0	68.05	2.22			63.93	2.12			
4	63.13	2.68	58.97	54.85	67.33	3.50			
8	69.43	2.93	64.25		69.55	3.06			
12	77.28	1.32			66.12	2.24	64.16		
16	75.62	2.39			67.76	1.51	72.80		
20	80.21	1.56			71.06	1.91	75.77	68.89	66.61

HSI

	Not fed				Fed				
Week	**Mean**	**SE**	**a**	**b**	**Mean**	**SE**	**c**	**d**	**e**
0	6.34	0.38			5.76	0.42			
4	4.53	0.58	5.00	6.35	5.69	0.57			
8	4.03	0.44	5.62		5.18	0.35			
12	2.56	0.23			5.07	0.60	5.96		
16	3.06	0.35			4.71	0.44	2.87		
20	2.19	0.50			6.34	0.25	6.69	6.15	5.88

Lipid content of the hepatopancreas

	Not fed				Fed				
Week	**Mean**	**SE**	**a**	**b**	**Mean**	**SE**	**c**	**d**	**e**
0	15.22	2.78			16.52	1.43			
4	17.00	2.58	21.06	24.98	15.82	3.54			
8	11.05	3.25	18.88		12.07	2.97			
12	5.63	2.02			18.03	2.22	17.6		
16	6.75	2.48			12.98	0.92	9.80		
20	2.58	1.32			11.79	1.76	8.32	15.03	15.72

C:N ratio of the tail muscle

	Not fed				Fed				
Week	**Mean**	**SE**	**a**	**b**	**Mean**	**SE**	**c**	**d**	**e**
0	3.16	0.04			3.18	0.01			
4	3.16	0.02	3.13	3.24	3.13	0.02			
8	3.20	0.14	3.77		3.15	0.03			
12	3.04	0.02			3.16	0.02	3.20		
16	3.01	0.02			3.15	0.02	3.19		
20	2.99	0.03			3.19	0.03	3.11	3.24	3.26

2.4 Discussion

N. norvegicus employs a range of internal mechanisms as a response to starvation. This was known prior to the present study, but only on the basis of one or two measures. It has been possible to show here that a much wider range of biochemical and biophysical changes occurs in *N. norvegicus* during starvation.

2.4.1 Effects of starvation on *N. norvegicus*

2.4.1.1 Phase 1

Copper in decapods is bound as prosthetic groups in the oxygen-binding haemocyanin protein molecules. Once a *N. norvegicus* is in a starved state then the haemocyanin begins to break down, resulting in a reduction in oxygen carrying capacity. Taylor & Anstis (1999) and Depledge & Bjerregaard (1989) noted storage of excess copper in the hepatopancreas, bound to metallothioneins. Results from this chapter show that the withdrawal of food from the animal does indeed induce the same effect, whereby the copper concentration in the haemolymph reduces for the first 12 weeks. This effect was shown by Baden *et al.* (1990), who recorded a 50% reduction in the haemocyanin concentration within 66 to 89 d of food starvation. Similar effects have been found in southern king crab, *Paralomis granulosa (*Comoglio *et al.*, 2005), the amphipod *Waldeckia obesa (*Chapelle *et al.*, 1994), and the Chinese shrimp, *Fenneropenaeus chinensis* (Zhang *et al.*, 2009). Baden *et al.* (1994) showed that the reduction in haemolymph copper concentration coincides with an increase in copper present in the hepatopancreas. With this association between decreases in copper and in haemocyanin during starvation, it can be speculated that the animal is entering a metabolically depressed state in which the rate of consumption of food and utilisation of internal metabolic reserves are both reduced, thus prolonging the period that the animal can survive without food.

2.4.1.2 Phase 2

The reduction in circulating haemocyanin could also reflect its use as a source of energy (Uglow *et al.*, 1986; Baden *et al.*, 1990) and so the possibility cannot be excluded that the reduction in haemocyanin concentration during starvation could reflect this process instead of, or as well as, indicating intrinsic metabolic depression (MD_i).

The present findings indicate that in *N. norvegicus*, glycogen in the tail and hepatopancreas along with haemolymph protein are the first reserves to be used, where from week 4 to 12 the glycogen declines in unfed individuals (Figure 2.4). It appears that once glycogen is depleted, the animal uses its other reserves such as lipids, both in the tail and the hepatopancreas.

In general, the protein content of the tail muscle did not decrease significantly over time in unfed animals, which is consistent with the results of Parslow-Williams (1998) and Mente *et al.* (2011) who both showed that within the time scale of 20 weeks there is no sustained fall in the protein content of the tail muscle.

In the present study lipids decreased from week 4 to week 12 in the hepatopancreas of unfed animals and the animals sampled at week 12 were the first group to be significantly lower than week 0 animals. Many other studies of decapods show a similar decrease in lipids under starvation. Examples include an Australian freshwater crayfish, *Cherax destructor* (Jones & Obst, 2000), the tiger prawn, *Penaeus esculentus* (Barclay *et al.*, 1983), and the Alaskan prawn, *Pandalus platyceros* (Whyte *et al.*, 1986). Even though a decrease in the lipid content of the hepatopancreas was observed in this study, Dall (1981) states that *N. norvegicus* did not utilise lipids within his starvation trial of 18 days, and indeed in the present study this was the same at that time point. It took 84 days (12 weeks), over four times longer than Dall's trial, to show a significant reduction in lipid content of the hepatopancreas. Mente (2010) also showed a significantly reduced hepatopancreas lipid content (12.16%) in *N. norvegicus* which had been starved for 8 months compared to animals fed on a pellet diet (56.47%) and a mussel diet (34.38%).

Dall (1981) and Barclay *et al.* (1983) argued that lipid alone is not sufficient for energy metabolism (or energy reserve), suggesting that lipids would last only 16 days for *P. esculentus* and 20 days for *N. norvegicus*. This is argued, even though over twice as much energy is derived from lipid catabolism when compared with carbohydrates (39.4 $kJ.g^{-1}$ and 17.6 $kJ.g^{-1}$, respectively) (Schmidt-Nielsen, 1997). Chang (1995), however, stated that the hepatopancreas is the major storage organ for energy reserves in crustaceans and Jones & Obst (2000) suggests that lipids are a major energy source in *C. destructor*, comprising 85% of the hepatopancreas energy reserves. Whyte *et al.* (1986) also saw a reduction in lipid content of the hepatopancreas in *P. platyceros* from 45.6% at day 0 to 18% at day 84. Storch *et al.* (1982) showed that the coconut crab *Birgus latro* can survive for one year without food, again using lipids as reserves.

Since, in the present study, glycogen seems to decrease significantly before lipids levels dropped significantly in unfed animals (which has also been shown in *C. destructor*; Jones & Obst, 2000), a switch seems to occur from utilising carbohydrate in the early stages of starvation to utilising lipids later. By this time, the reduction of copper in the haemolymph seems to slow and beyond week 12 there is no further reduction in copper, suggesting that this is the time point when the metabolism cannot be depressed any further. Also, lipids can only be catabolised in the presence of oxygen, needing 2 L $O_2.g^{-1}$ of lipid, compared with 0.84 L $O_2.g^{-1}$ for carbohydrate (Schmidt-Nielsen, 1997). Therefore, the animal may not depress its metabolism any further when the glycogen has been depleted, as the oxygen demand would be too great. However, as already stated above, lipids produce twice as much energy than carbohydrates per unit weight, so this may not be the case. The water content of the hepatopancreas then increases, corresponding to the reduction in lipids. As lipids in a tissue are catabolised, metabolic water is produced in almost the ratio 1:1 (1.07 g H_2O for every gram of lipid catabolised; Schmidt-Nielsen, 1997). This water and/or external water collects in the tissue to replace some of the mass and maintain body volume and internal turgidity (Comoglio *et al.*, 2005). The weight as shown in the CL: weight ratio did not reduce throughout the trial. The present findings suggest that the proportion of the combined lipid and water content of the hepatopancreas tends to be around 80% of the hepatopancreas (Figure 2.15a). The overall mass decrease in the hepatopancreas, and the

changes in the proportions of different constituents during reduced nutritional status, can been seen in Figure 2.15b.

The analysis of the lipid contained within the tail muscle indicates that it decreases as the animal goes through periods of starvation. Semenina & Tiunov (2011) and Haubert *et al.* (2005) show that in springtails (Collembola), the lipid content of a tissue is negatively correlated with the C:N ratio. This relationship has also been found in the present study. The tail muscle however does not seem to reflect the dietary stresses of the animal to the same extent as the hepatopancreas. This corroborates the findings of Jones & Obst (2000) and McLeod *et al.* (2004), who state that the hepatopancreas is the central site of digestion and the store of major nutrient reserve in various species of decapods. The water content in the tail does not increase significantly, indicating that lipid levels in the tail are also unlikely to change to a great extent. The amount of lipid contained in the tail muscle is very small (*ca* 1% of the tail mass) and is therefore not likely to be a substantial reserve to sustain the lobster. Mente *et al.* (2011) stated that the tail muscle made up *ca* 22.1% of the total body weight (with claws). If the lipid content of the tail muscle is converted to total weight in the tail, the fed group had a mean value of 0.095 g of lipids within the tail, whereas the unfed group had a mean value of 0.055 g of lipids in the tail. This is a drop of 0.04 g due to food deprivation. Schmidt-Nielsen (1997) states that lipids provide 39.4 $kJ.g^{-1}$ when catabolised, suggesting that the tail lipid loss throughout this experiment contributed *ca* 1.576 kJ of energy through the period of starvation. In comparison, the fed group at week 20 had a mean of 0.21g of lipids in the hepatopancreas and the unfed group at week 20 had a mean 0.03 g of lipids in the hepatopancreas. This amounts to a utilisation of 0.18 g, yielding 7.092 kJ of energy. Parslow-Williams *et al.* (2002) showed that the minimum maintenance ration of quiescent (metabolically deprived) animals was 0.27 $kJ\ d^{-1}$ (when held at 10˚C), suggesting that the lipids contained in the tail muscle could sustain a metabolic depressed quiescent animal for *ca* 6 days and the lipids contained in the hepatopancreas could sustain the same animal for *ca* 26 days.

It is well documented that the stable isotope ratio of $\delta^{15}N$ increases with starvation in other animals, as amino acids derived from protein breakdown are catabolised (Haubert *et al.*, 2005) and progressively enriched in ^{15}N through

breakdown of existing tissue protein. However, as Parslow-Williams *et al.* (2001) and this study implied, protein is not catabolised in *N. norvegicus* as their primary source of energy. Therefore the isotopes within the tail muscle (or the hepatopancreas) are not likely to be altered in the ways expected in the unfed group of animals. This conclusion is supported by the lack of change in nitrogen isotope ratios within the tail muscle.

There have also been conflicting reports of whether stable isotope changes indicative of starvation (an increased δ^{15}N signature) actually occur. McCue & Pollock (2008) review various studies; some organisms such as a species of quail, show enrichment between 0.5‰ and 1.5‰ in different tissues of the bird, whereas the mysid shrimp *Neomysis integer* showed no significant difference in either δ^{15}N or δ^{13}C values over a 5 week starvation trial. This suggests that many light isotopes are used up in catabolism. McCue & Pollock (2008) indicate that, in the studies they reviewed, uric acid and urine always show enrichment in the heavier nitrogen isotope thus increasing δ^{15}N, even when in the same study muscle or other tissues were not enriched. These nitrogenous waste products were not measured in the present study.

Tail δ^{15}N showed an increase in fed animals sampled at weeks 16 and 20, compared with animals sampled earlier in the trial, and to unfed individuals in weeks 16 and 20. This could be due to these animals reflecting (or equilibrating) the δ^{15}N relative to the food item (squid at *ca* 14‰). This is an interesting finding in itself as a feeding equilibrium has never, to the knowledge of the author, been determined for any decapod species. This result shows that 16 weeks could be when *N. norvegicus* would start reflecting a new diet and equilibrating to its new trophic position. However, much more work would need to be carried out to determine the validity of this.

To summarise, starved *N. norvegicus* males enter an increasingly depressed metabolic state for the first 12 weeks. Within this time glycogen stores, and possibly protein stores from the haemolymph in the form of haemocyanin, are utilised. By week 12, catabolism has significantly reduced lipid levels in the hepatopancreas; however the levels can be seen to decrease from week 4. The tail muscle changes very little over the study period, with only glycogen and a

small amount of lipid being lost from this large tissue mass. There seems to be no overall weight change in *N. norvegicus*. However, the weight of the hepatopancreas does seem to decrease, as indicated by the decrease in HSI. The mass balance is likely to be made up of water within the cephalothorax from the catabolism of lipids and/or from intake of sea water.

2.4.2 Could these measures be used with field samples?

As seen in Table 2.1 there were seven measures which showed a clear significant difference between week 0 and week 20 in unfed animals by the end of the trial. These differences were shown in the hepatopancreas (copper, lipid, glycogen, HSI and water), haemolymph (copper) and in the tail muscle (C:N ratio). However, only five measures were selected: HSI, hepatopancreas water, copper content of the hepatopancreas, lipid content of the hepatopancreas and the C:N ratio of the tail muscle, as they fulfilled the three criteria set out at the start of the trial. Namely, for each measure:

1. There is a clear difference between fed and unfed animals.
2. This difference remains beyond a certain time point.
3. It is insensitive to short term stresses caused by the method of collection (e.g. trawl capture).

2.4.2.1 Measures that seem unsuitable for use as biomarkers

Glycogen

The carbohydrate store does decrease in starved *N. norvegicus.* However, it is also sensitive to short term stresses caused by the method of collection, thus not fulfilling criterion 3. Albalat *et al.* (2009) showed that, during trawl capture, *N. norvegicus* uses the majority of its glycogen stores in the stress response to capture. This response includes the vigorous tail flipping action as the animal is trying to avoid capture. Albalat *et al.* (2010) also showed that *N. norvegicus* were able to increase their glycogen stores subsequently when held in a recovery tank or within an aquarium. It seems that this recovery period could actually last for at least 9 weeks as, in the present work, animals at the 4 week point in the trial (which was 9 weeks from capture, due to the initial 2 week acclimatisation period) had increased glycogen, compared with animals at capture. Therefore,

due to the long-term effects of trawl capture, glycogen would not provide a reliable measure of starvation in field-caught animals.

Haemolymph copper
Haemolymph copper was not selected, as levels stabilised beyond 12 weeks. Also, there was also a lot of variation in the data.

2.4.2.2 Significant measures that could indicate degree of starvation

Hepatopancreas copper
Copper showed the greatest changes of all the measures of starvation, increasing throughout the trial in the hepatopancreas of unfed animals. Up to week 12 this effect was strong, which validates the use of copper as a biomarker of starvation. A reduction in this trend at week 20 was probably a sampling effect, Further work is necessary to fully evaluate further changes beyond week 12, in order to determine the limits of copper accumulation in the hepatopancreas.

HSI
The HSI provides an effective biomarker to measure the nutritional status of *N. norvegicus*. The present results show a clear significant reduction in the index from week 8 onwards. Jones & Obst (2000) determined HSI and referred to six other studies that have also used this measure for nutritional status studies. Assessment of HSI has repeatedly been shown to be robust and can be used to assess a large number of animals rapidly, simply and cheaply.

Hepatopancreas water
Hepatopancreas water content is also a cheap and easy biomarker to use. The present results show a clear significant increase in this measure in unfed individuals from week 12 onwards.

Tail muscle C:N ratio
The tail muscle C:N ratio was the only useable measure found in the tail, decreasing as a response to starvation. However, as this measure is based on stable isotope analysis it is more involved, and quite expensive. The costs could be reduced by measuring the amount of carbon and nitrogen directly. The tail muscle C:N ratio gives a measure which is independent of the hepatopancreas. If it is possible to use biomarkers in both major tissues (tail and hepatopancreas)

of *N. norvegicus*, then more confidence could be placed in a putative starvation response, by compensating for possible experimental error in one measure.

Hepatopancreas lipid

Hepatopancreas lipids fulfil all the desired criteria, showing a significantly detectable difference between fed and unfed individuals at week 12 onwards, with lipids decreasing in the hepatopancreas over time in unfed animals. This is a medium-cost biomarker which shows biochemical changes in the animal.

2.4.3 Factors that could affect these measures in the field

2.4.3.1 Moulting

As Chang *et al.* (1995) expressed it, "the physiology and biochemistry of the crustacean change dramatically during the moult cycle". Therefore any study using these biomarkers needs to take moult cycle variations into account. Chang *et al.* (1995) allude to studies that show that lipids will increase in the premoult stages in crayfish, land crabs and shrimp. However, Alvarrez-Fernadez *et al.* (2005) showed that in *N. norvegicus*, lipid levels reduce in early premoult and then increase to the same level as intermoult at later premoult stages, but then decrease again at postmoult. Haemocyanin can also be lower in postmoult and then rise in premoult reflecting a higher metabolic activity at this stage (Chang *et al.,* 1995; Hagerman, 1983). These changes can be attributed to biochemical changes associated with moulting (Heath & Barnes, 1970), such as absorbing water to increase the volume of the soft body (Aitkin, 1980), changes in oxygen consumption (Alcaraz & Sarda, 1981), change in enzyme activity (Fernandez *et al.*, 1997), formation of calcium carbonate in the form of gastroliths (Gramitto, 1998) and also the cessation of feeding due to lack of appetite (Zhang *et al.*, 2009). In the present study only a few animals went through ecdysis during the course of the trial. Gastroliths are present for 15 d premoult in *N. norvegicus* and are then reabsorbed around 24 h after ecdysis (Gramitto, 1998). Starvation seemed to delay moulting, i.e. more fed animals moulted than starved ones. This starvation-induced delay in moulting was also shown in *Carcinus maenas* (Hagerman, 1983) as well as in two independent 6-month starvation studies in *N. norvegicus* where lower moulting rates occurred in starved animals (present

study and H. Philp pers. comm.). As moulting is known to affect these measures, subsequent chapters will refer only to results on intermoult animals.

2.4.4 Other starvation studies

It seems that a number of different strategies are employed by different crustaceans when they are subjected to starvation. Work on both the brown shrimp, *Crangon crangon* (Regnault, 1981), and the crab *Carcinus maenas* (Heath & Barnes, 1970) showed that lipids are utilised first, whereas *F. chiensis* (Zhang *et al.*, 2009) and *N. norvegicus* (Dall, 1981; and this present study) do not appear to utilise lipids first. Therefore, as McLeod *et al.* (2004) state, care needs to be exercised when generalising about the mechanisms of crustacean responses to starvation. Work such as that carried out in the present study is necessary before selecting appropriate biomarkers to use on field-samples animals.

Table 2.3 shows how different species of crustacean react to starvation and indicates which measures and timescales have been utilised in the different studies. All studies that measured metabolic rate (either directly or indirectly) showed a decrease during early stages of starvation, apart from that on *Pandalus platyceros* (Whyte *et al.*, 1986), in which the metabolic rate remained constant for 61 days, while lipids seemed to increase in that period. All but Dall (1981) showed a decrease in lipids. The results on protein utilisation were variable.

A few other methods have been used to monitor the responses of crustaceans to the lack of food. The O:N ratio measures the amount of oxygen consumed in relation to the amount of nitrogen excreted. Protein catabolism results in a lower O:N ratio than catabolism of lipids, due to the lower quantity of oxygen required to liberate the same amount of energy from protein and the greater quantity of nitrogenous waste produced. The O:N ratio has been used by Chapplle *et al.* (1994), Comogilo *et al.* (2005) and Zhang *et al.* (2009), but it only gives an indication of the source of catabolism, so it is not an adequate biomarker for use in the field. However, the method could prove useful for future laboratory trials to note what nutritional state the animal is in. Also, as the method is non-invasive, it can be used to monitor a single animal throughout its starvation period.

A molecular measure related to nutritional status, the RNA:protein ratio, has been determined in many species, including *N. norvegicus*, as reviewed by Parslow-Williams *et al.* (2001). Other methods include enzyme synthesis (Regnault, 1989), gut fullness (Sarda & Valladares, 1990), ATP synthesis (Dickson & Giesy, 1982), and gastric fluid constituents (Dall, 1975).

One field which has been developed over the last few decades is that of 'omics' (transcriptomics, metabolomics, genomics, proteomics): here every constituent is measured (metabolites for metabolomics, genes for genomics etc.). These have the potential to provide extensive information about the metabolic adaptations that occur during starvation responses, and offer much greater sensitivity to detect the earliest expression of these.

Table 2.3 Summary of other studies investigating starvation in crustaceans.

Species	Ref	length (days)	Holding Temp. (°C)	Metabolic depression	Lipids
Cherax destructor	Jones & Obst, 2000	~112 (16 wk)	25 & 10	N/A	decline after week 4
Crangon crangon	Regnault, 1981	30	12-14	reduced oxygen consumption by 10% over first 10days; 42% drop until the end of experiment	N/A
Fenneropenaeus chinensis	Zhang *et al.*, 2009	24	25.5 - 26.5	drop in Oxygen consumption first 4 days then remains steady	N/A
Jasus edwardsii	McLeod *et al.*, 2004	28	16-19	N/A	Muscle different in starved at day 28. Hepatopancreas significant at day 14 and 28
N. norvegicus	Baden *et al.*, 1994	~210 (7 mo)	8 - 10	Drop in Haemocyanin after 12 days, 13% drop after 7 months. No change the concentration of copper in the muscle tissue. Hepatopancreas up to 1550 $\mu g.g^{-1}$ copper after 7 mo, haemolymph copper low as 30 $\mu g.g^{-1}$	N/A
	Dall, 1981	18	11-13	N/A	no drop in 18 days
	Parslow-Williams, 1998	84	10	N/A	N/A
	Mente, 2010	~ 230 (8 mo)	10-12	N/A	Hepatopancreas: Starved -12.16% Feed pellet – 56.47% Feed mussel- 34.38%
	this study	140	11.7	Reduction in copper over the first 12 weeks in the haemolymph which is also seen as an increase in copper in the hepatopancreas, levels off in line with lipid catabolism	Significant decrease by week 12
Pandalus platyceros	Whyte *et al.*, 1986	84	8.5	steady until day 61 then declines	initial increase to day 22 then decreases to day 84
Paralomis granulosa	Comoglio *et al.*, 2005	12	7.5 - 8.5	Oxygen consumption decreased until week 9.	decrease after week 9 when Oxygen increases again
Penaeus esculentus	Barclay *et al.*, 1983	21	21 - 23	N/A	drops after 7 days, down to 1% after 14 days loss of 84mg
Waldeckia obesa	Chapelle *et al.*, 1994	60	-0.5 - 0.5	Oxygen consumption decreased over first 5 days increased up to day 25 then decreased again for the rest of the study	proxy measured via O: N ratio- both seem to decrees

Table 2.3 cont.

Species	Ref	Protein	Glycogen	other
Cherax destructor	Jones & Obst, 2000	decline after week 2	N/A	H.S.I decreases however lower temperature slows the rate of decrease down
Crangon crangon	Regnault, 1981	N/A	N/A	N/A
Fenneropenaeus chinensis	Zhang *et al.*, 2009	N/A	N/A	N/A
Jasus edwardsii	McLeod *et al.*, 2004	no significant difference found	no significant difference found	no significant difference found in ash content and energy stores.
N. norvegicus	Baden *et al.*, 1994	N/A	3% lower in muscle, 10% lower in hepatopancreas in relation to fed animals	N/A
	Dall, 1981	N/A	not measured	Water no increase after 18 days
	Parslow-Williams, 1998	no significant difference found	N/A	DNA different in hepatopancreas, RNA: protein ratio different in muscle
	Mente, 2010	N/A	N/A	N/A
	this study	No significant difference found	declined by week 12	HSI decrease, water content of hepatopancreas increase and others see text
Pandalus platyceros	Whyte *et al.*, 1986	N/A	N/A	N/A
Paralomis granulosa	Comoglio *et al.*, 2005	first decreases week 6	N/A	N/A
Penaeus esculentus	Barclay *et al.*, 1983	drops after 7 days, down to 10.6% after day 14, loss of 550mg	N/A	whole body water increased as protein and lipids decreased
Waldeckia obesa	Chapelle *et al.*, 1994	N/A	N/A	N/A

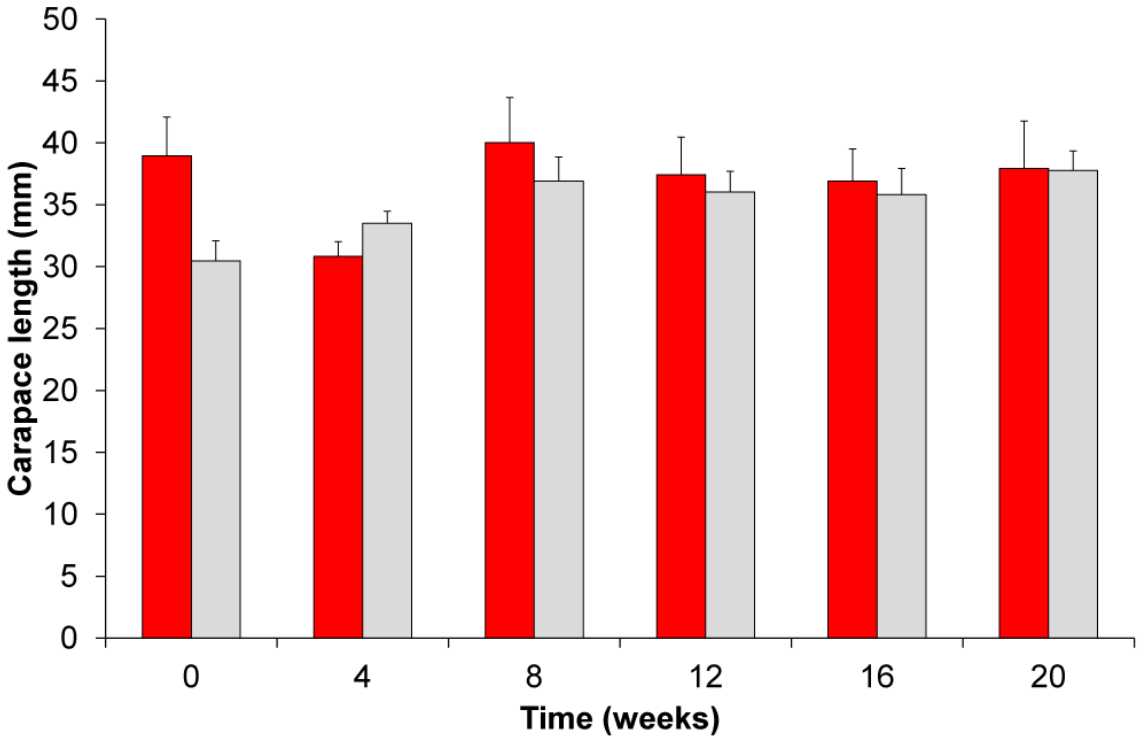

Figure 2.2 Carapace length (means ± SE) of unfed (red bars) and fed (grey bars) groups of male *N. norvegicus* during the starvation trial, time point 0 is the start of food deprivation. n=60

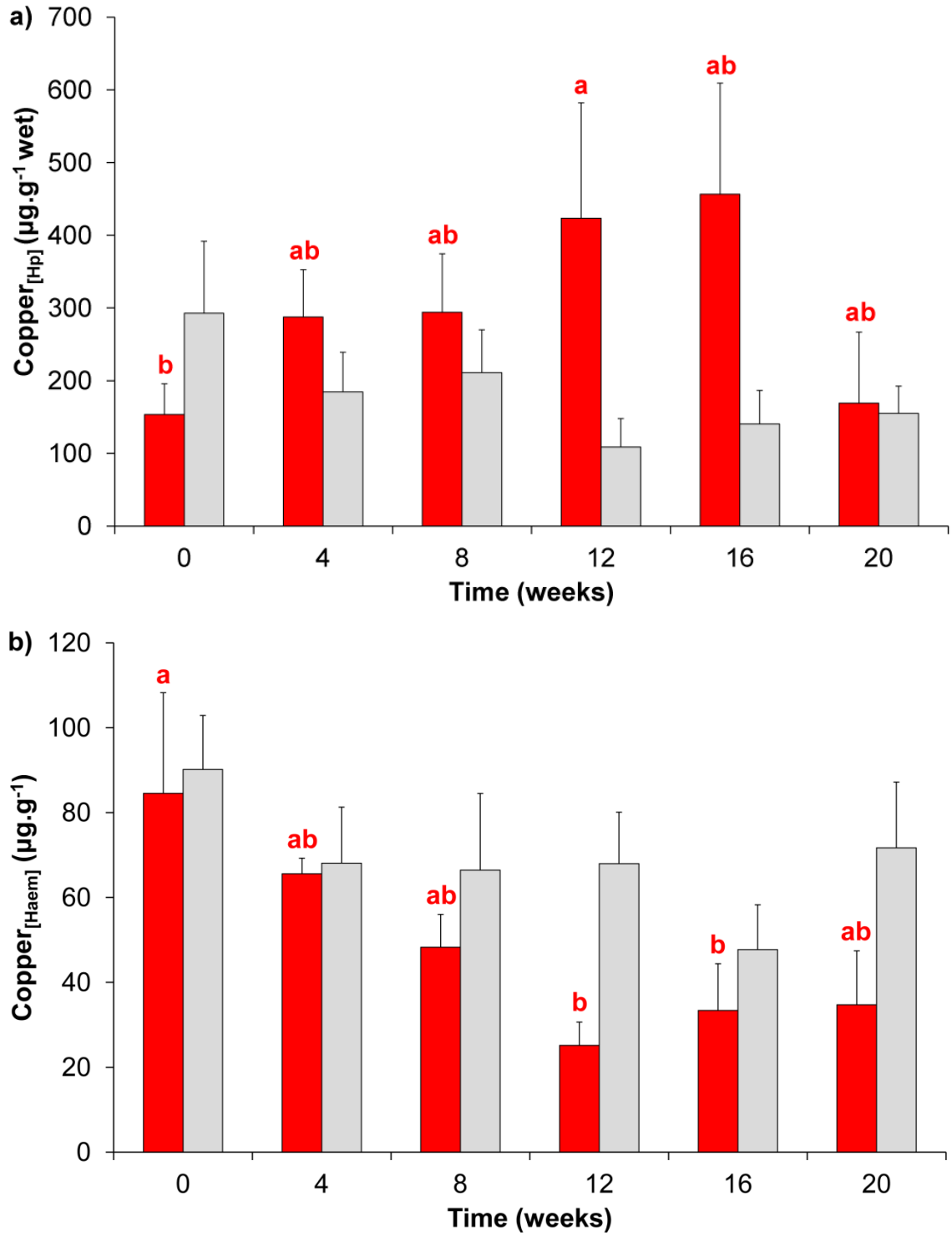

Figure 2.3 Copper concentration of a) hepatopancreas b) haemolymph (means ± SE expressed as wet weights) in unfed (red bars) and fed (grey bars) groups of male *N. norvegicus* during the starvation trial. Time point 0 is the start of food deprivation. Red letters indicate the results of a post hoc Tukey test on unfed groups. Means that do not share a letter are significantly different ($P<0.05$). n=55

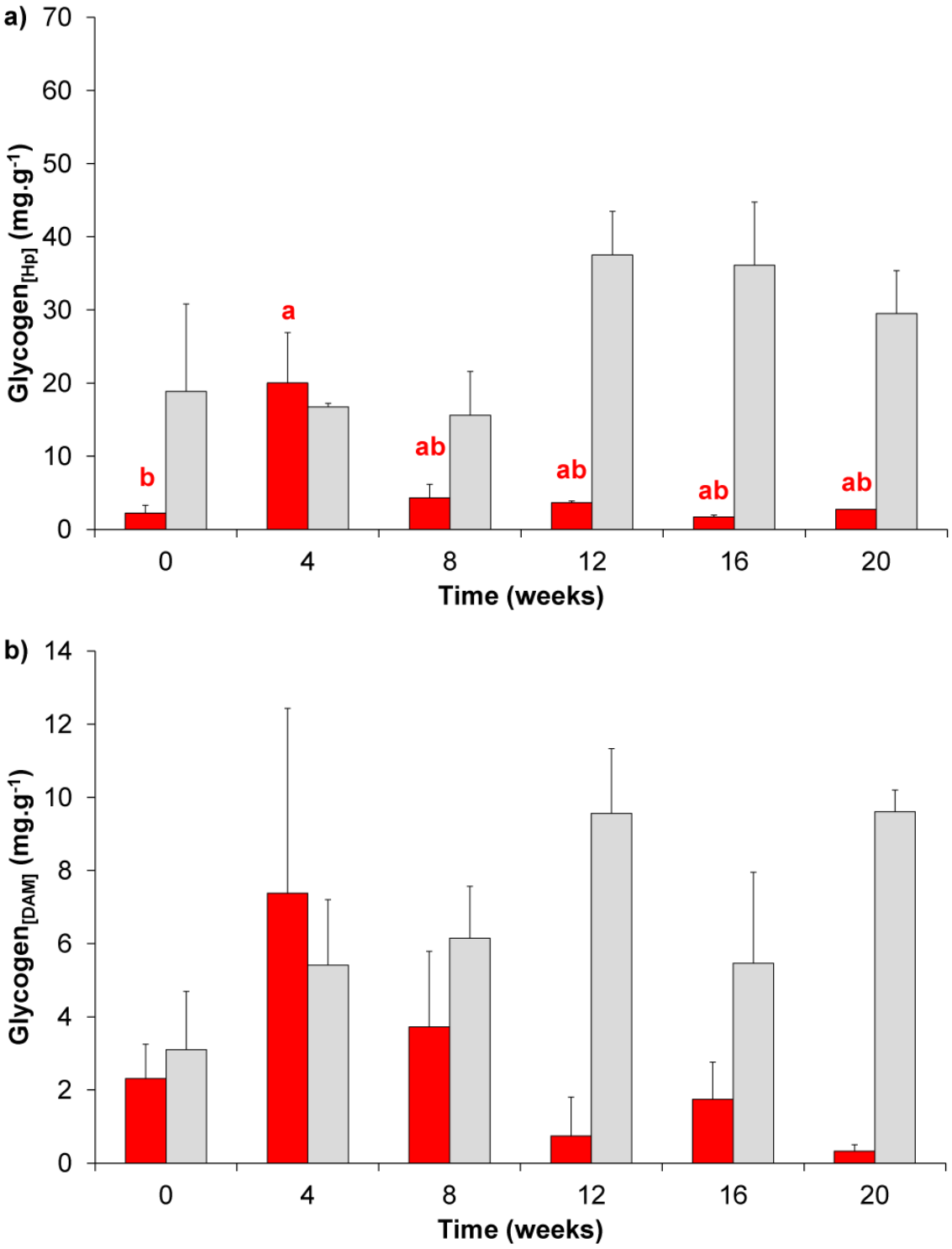

Figure 2.4 Glycogen concentration of a) hepatopancreas and b) tail muscle (means ± SE expressed as wet weights) in unfed (red bars) and fed (grey bars) groups of male *N. norvegicus* during the starvation trial. Time point 0 is the start of food deprivation. Red letters indicate the results of a post hoc Tukey test on unfed groups. Means that do not share a letter are significantly different ($P<0.05$). a) n=46, b) n=58.

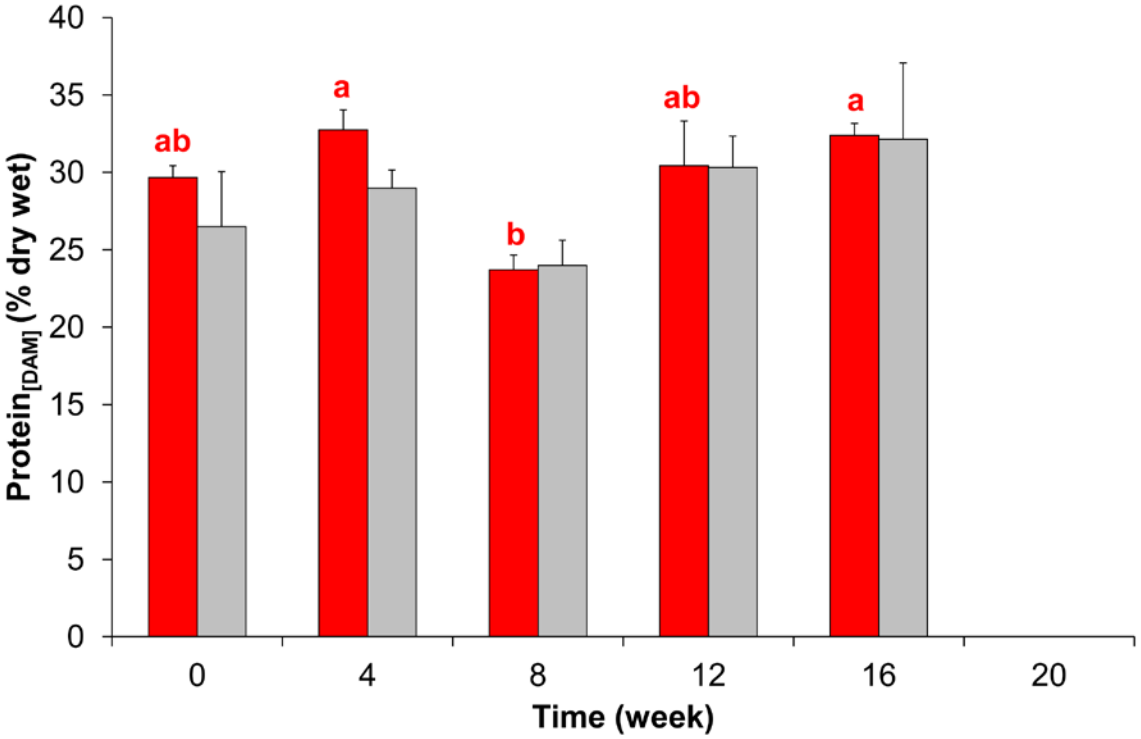

Figure 2.5 Protein content of the tail muscle (means ± SE) in unfed (red bars) and fed (grey bars) groups of male *N. norvegicus* during the starvation trial. Time point 0 is the start of food deprivation. Red letters indicate the results of a post hoc Tukey test on unfed groups. Means that do not share a letter are significantly different ($P<0.05$). n=52

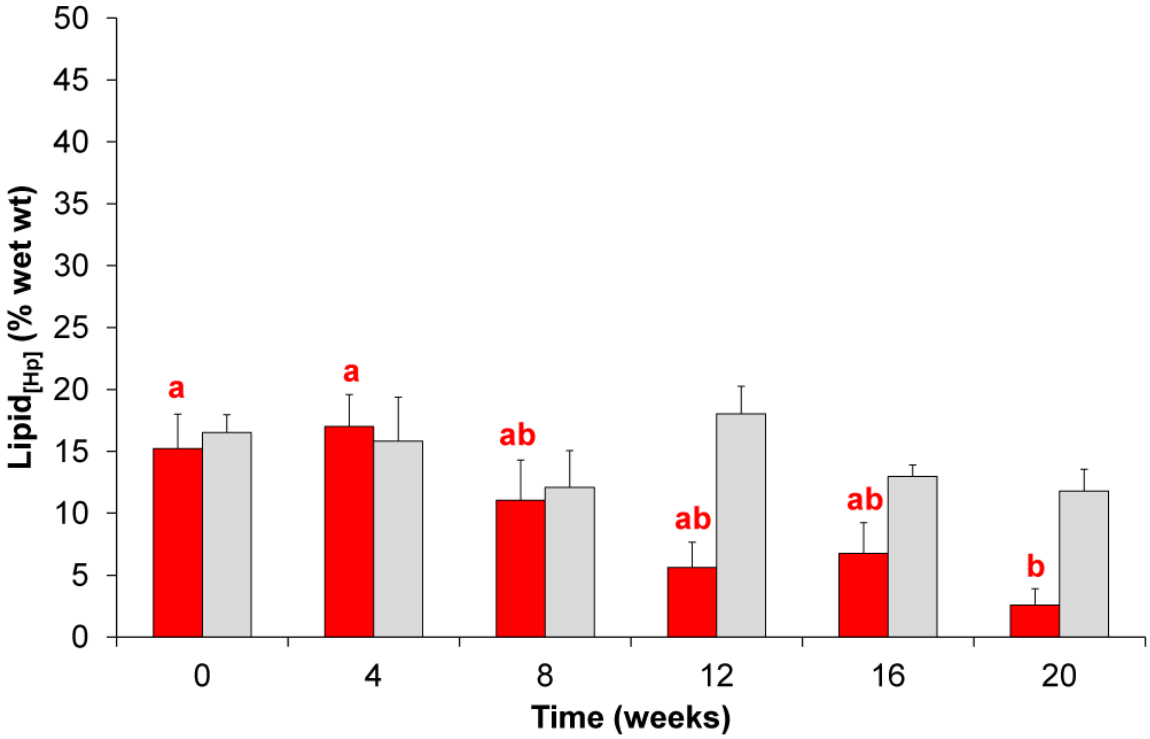

Figure 2.6 Lipid concentration of the hepatopancreas (means ± SE expressed as wet weights) in unfed (red bars) and fed (grey bars) groups of male *N. norvegicus* during the starvation trial. Time point 0 is the start of food deprivation. Red letters indicate the results of a post hoc Tukey test on unfed groups. Means that do not share a letter are significantly different ($P<0.05$). n=51

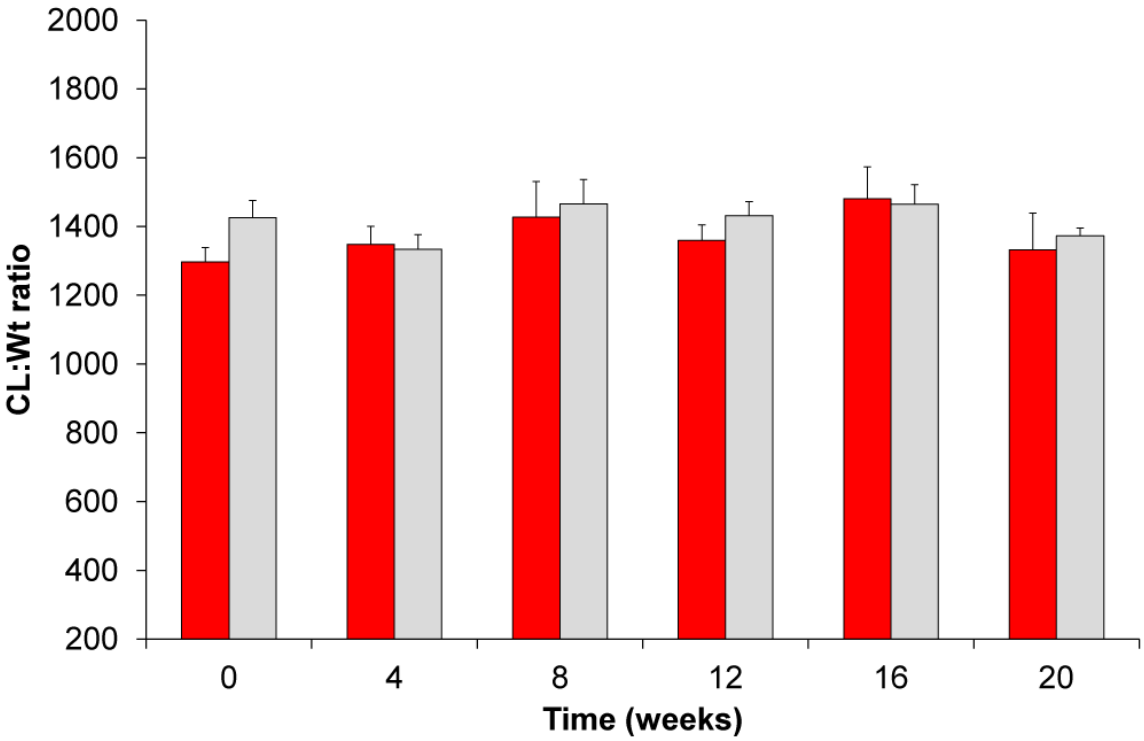

Figure 2.7 Carapace length: weight ratio (means ± SE) in unfed (red bars) and fed (grey bars) groups of male *N. norvegicus* during the starvation trial. Time point 0 is the start of food deprivation. n= 60

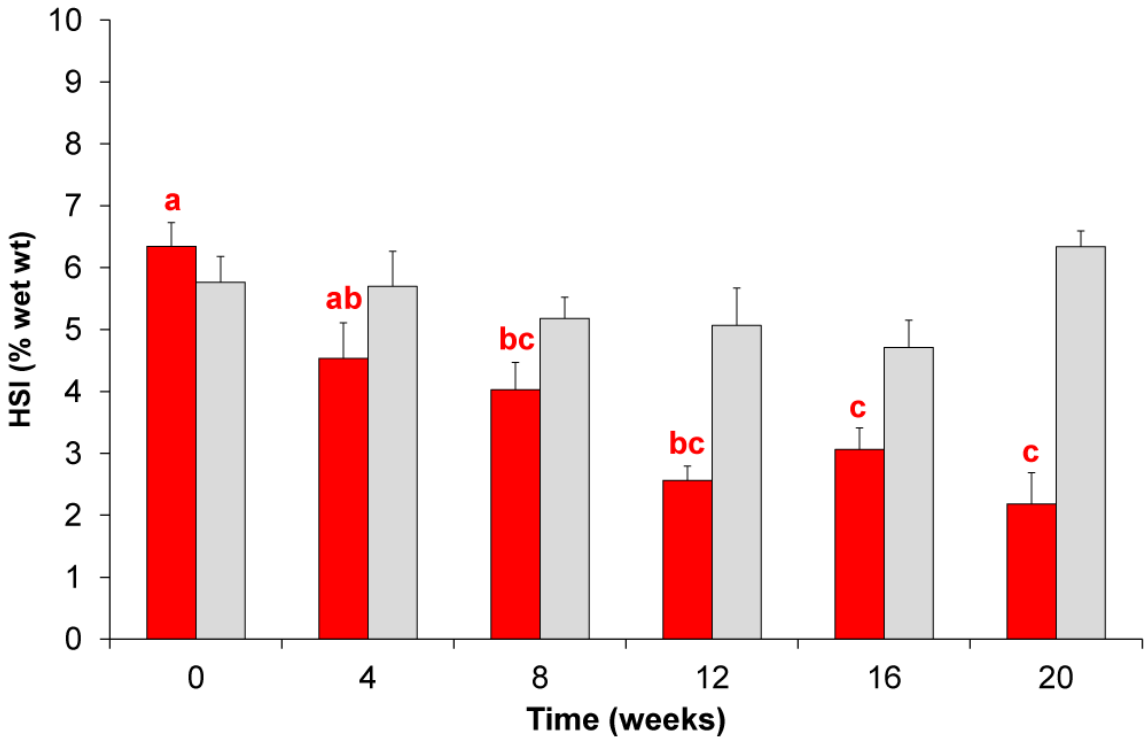

Figure 2.8 Hepatopsomatic index (HSI) (means ± SE) in unfed (red bars) and fed (grey bars) groups of male *N. norvegicus* during the starvation trial. Time point 0 is the start of food deprivation. Red letters indicate the results of a post hoc Tukey test on unfed groups. Means that do not share a letter are significantly different ($P<0.05$). n=61

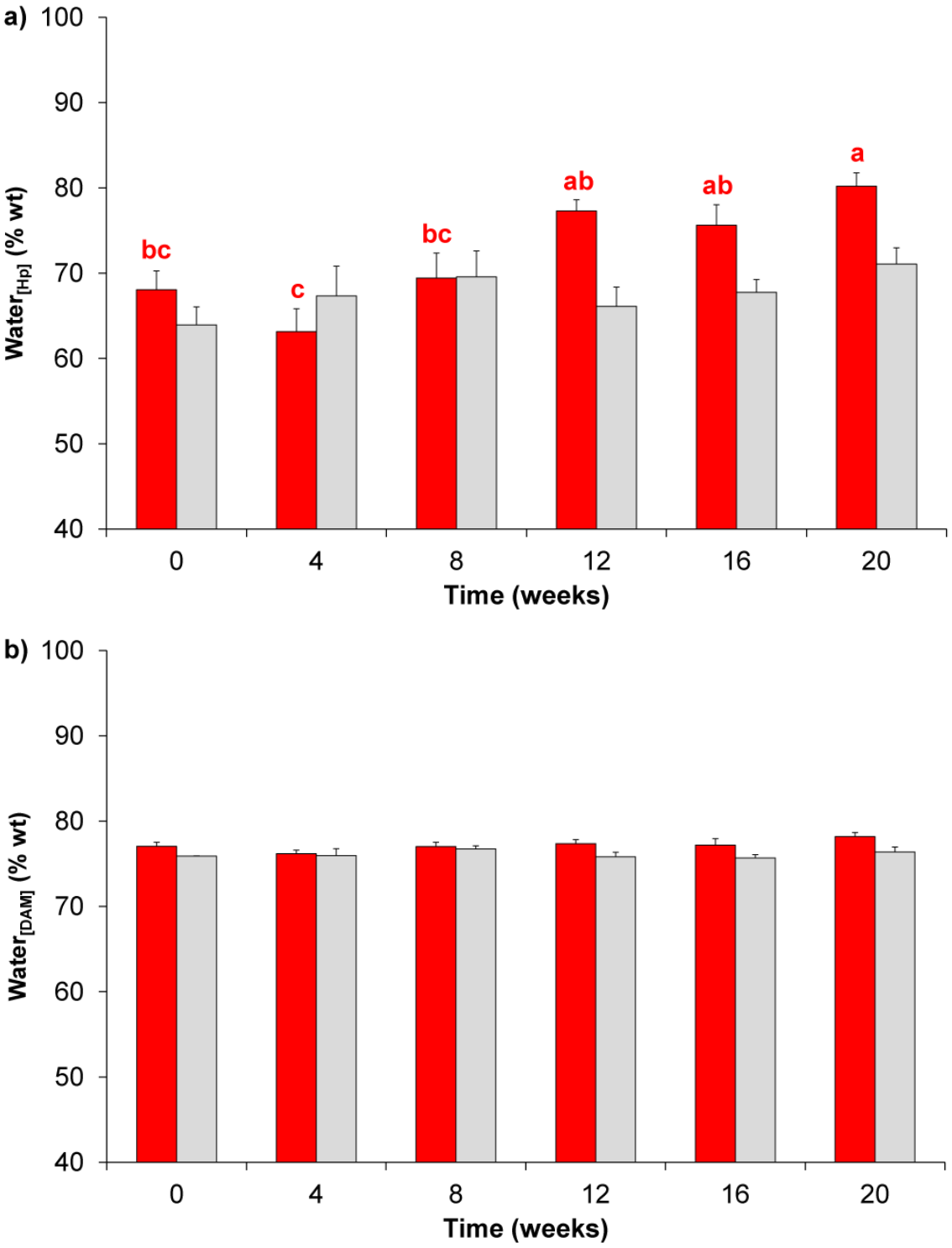

Figure 2.9 Water concentration of a) hepatopancreas and b) tail muscle (means ± SE) in unfed (red bars) and fed (grey bars) groups of male *N. norvegicus* during the starvation trial. Time point 0 is the start of food deprivation. Red letters indicate the results of a post hoc Tukey test on unfed groups. Means that do not share a letter are significantly different ($P<0.05$). n=61

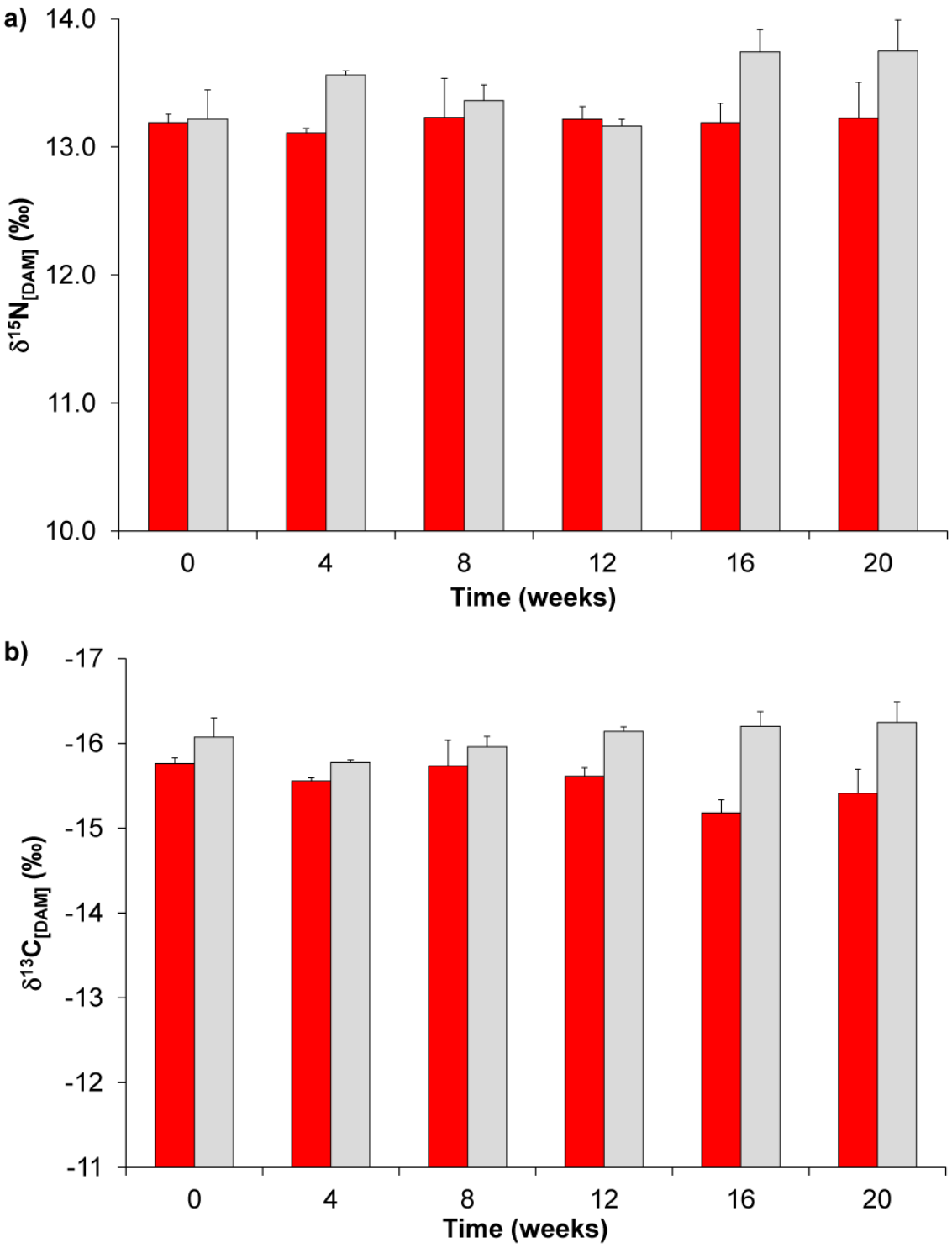

Figure 2.10 Stable Isotope of a) δ^{15}N and b) δ^{13}C in tail muscle (means ± SE) in unfed (red bars) and fed (grey bars) groups of male *N. norvegicus* during the starvation trial. Time point 0 is the start of food deprivation. n=61

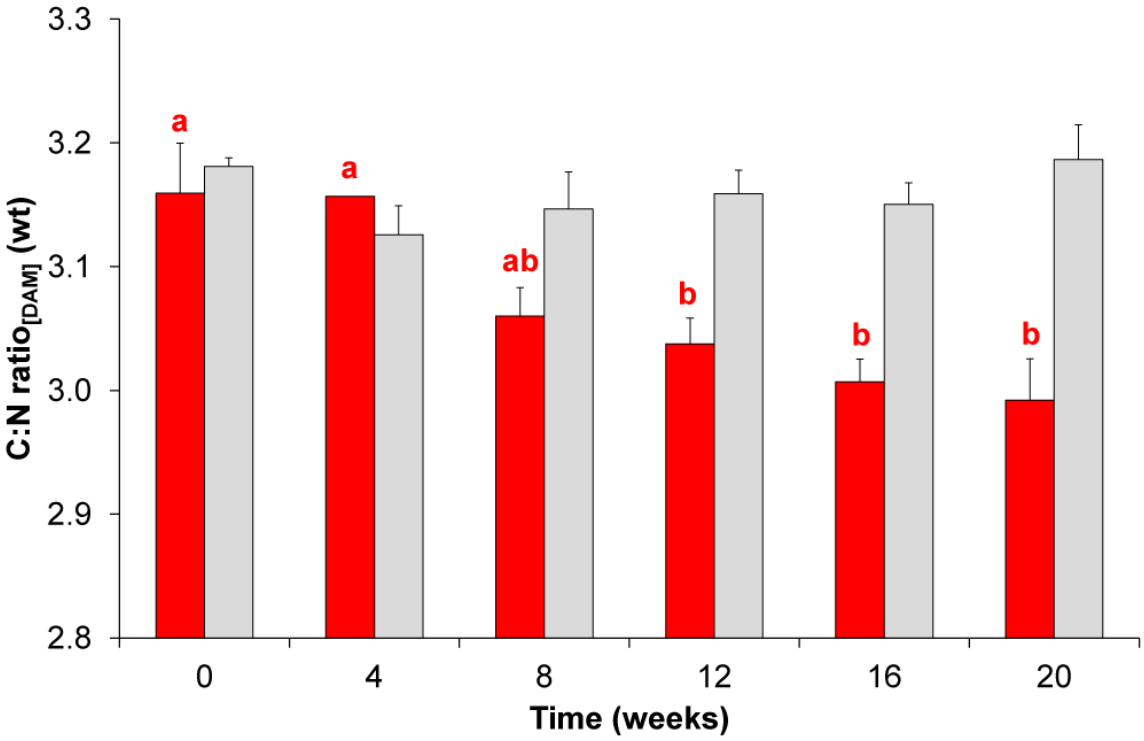

Figure 2.11 Carbon:Nitrogen ratio (C:N) of the tail muscle (means ± SE) in unfed (red bars) and fed (grey bars) groups of male *N. norvegicus* during the starvation trial. Time point 0 is the start of food deprivation. Red letters indicate the results of a post hoc Tukey test on unfed groups. Means that do not share a letter are significantly different ($P<0.05$). n=61

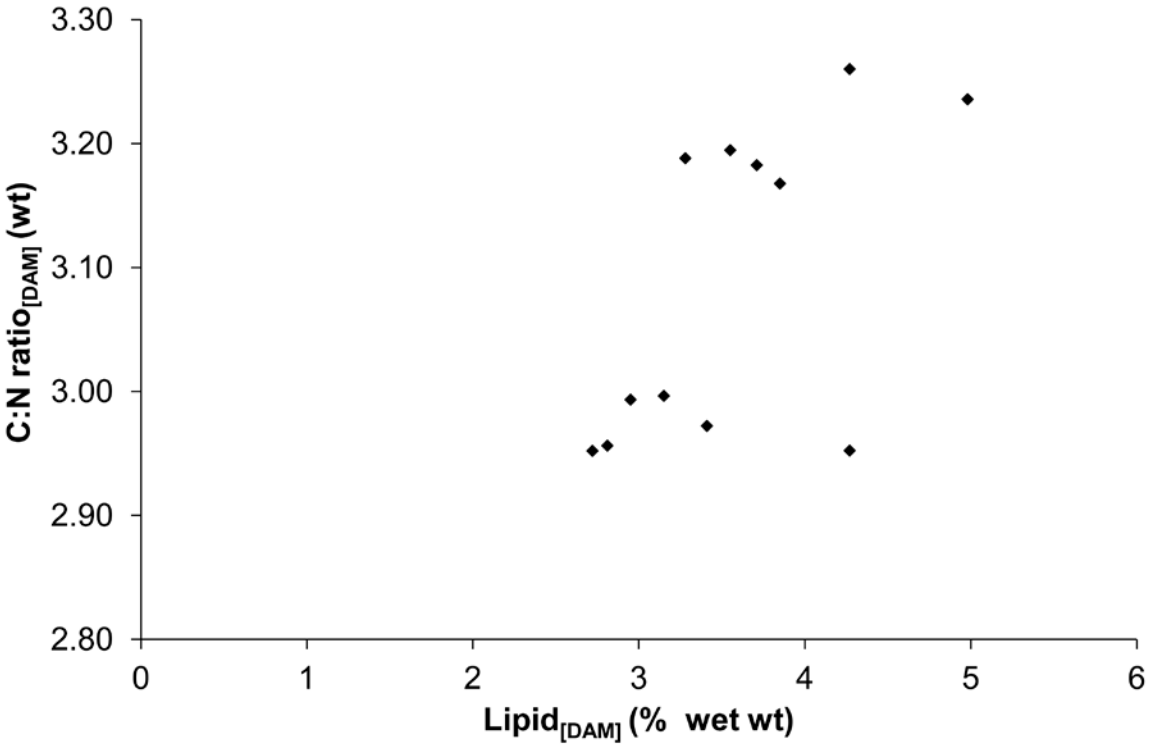

Figure 2.12 Carbon Nitrogen C:N ratio of tail muscle compared with lipid concentration unfed animal tails. n=12

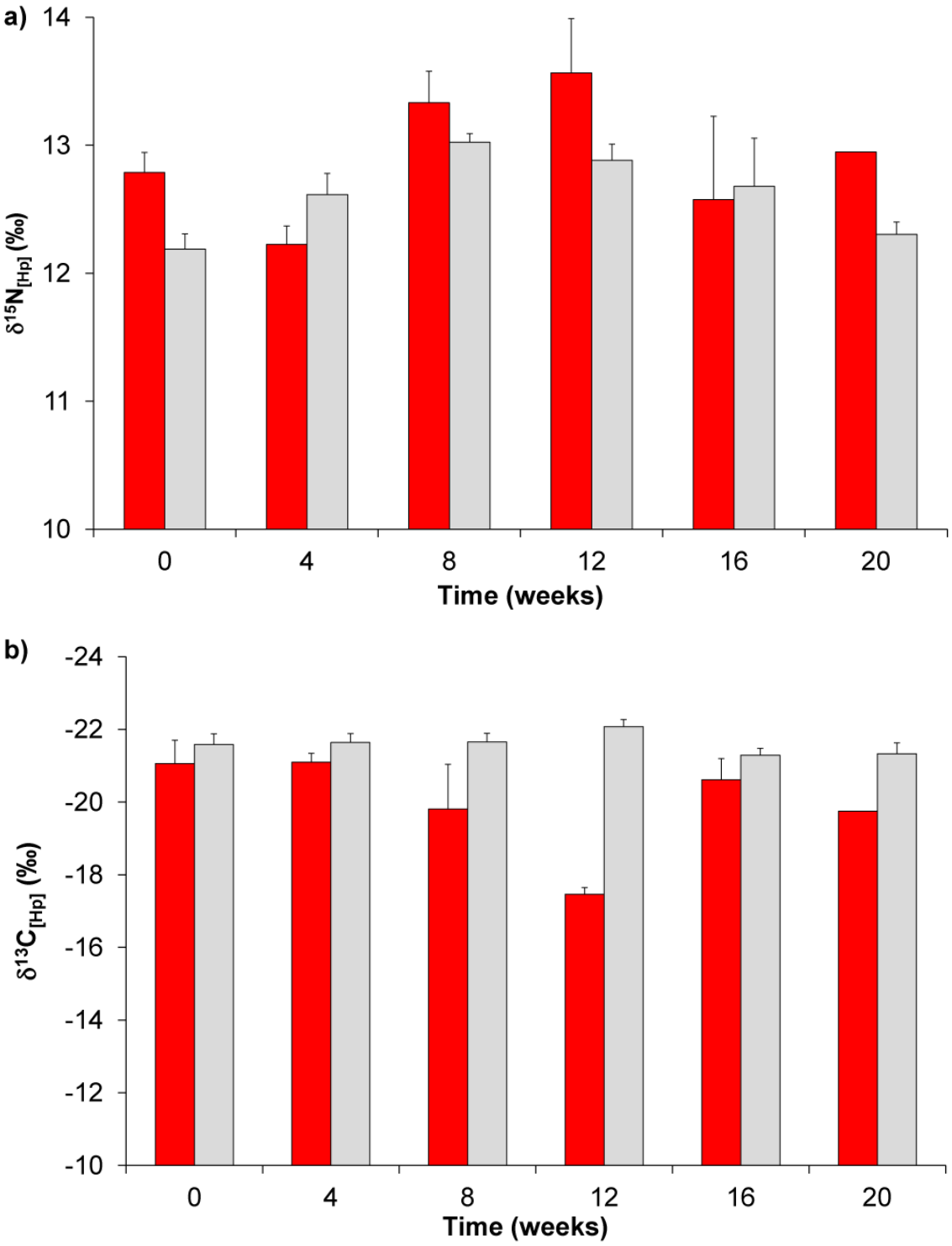

Figure 2.13 Stable Isotope of a) δ^{15}N and b) δ^{13}C in hepatopancreas (means ± SE) in unfed (red bars) and fed (grey bars) groups of male *N. norvegicus* during the starvation trial. Time point 0 is the start of food deprivation. n=34

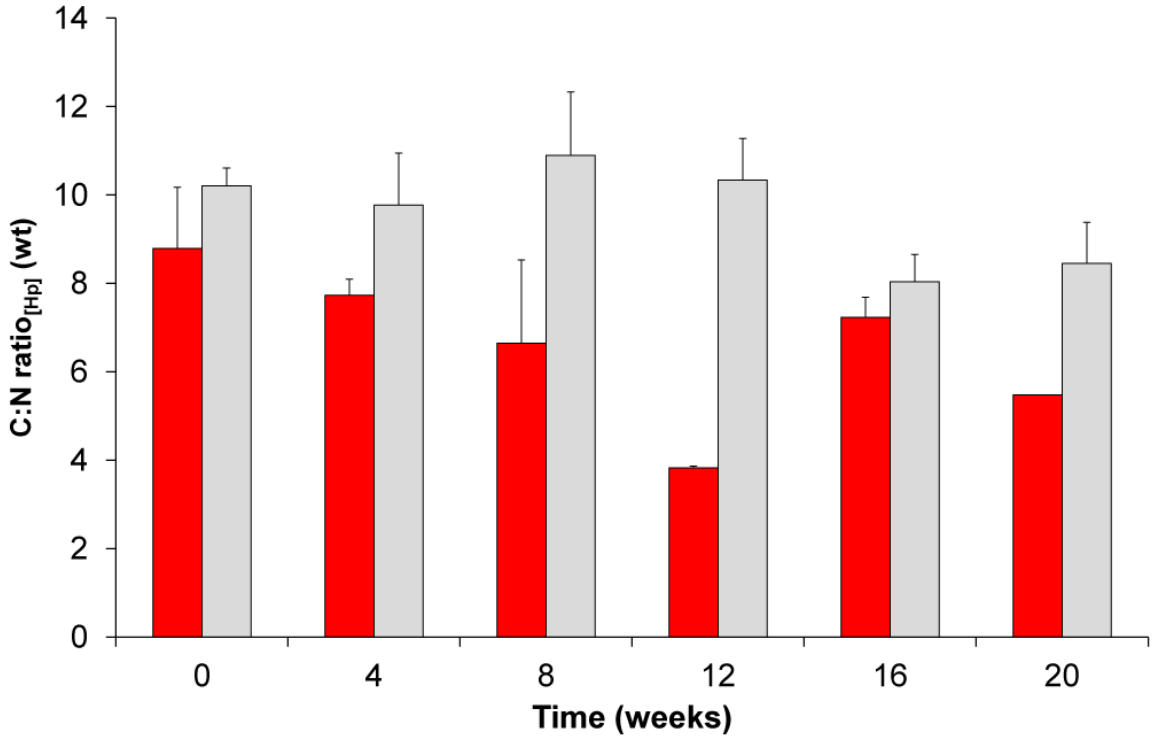

Figure 2.14 Carbon:Nitrogen ratio (C:N) of the hepatopancreas (means ± SE) in unfed (red bars) and fed (grey bars) groups of male *N. norvegicus* during the starvation trial. Time point 0 is the start of food deprivation. n=34

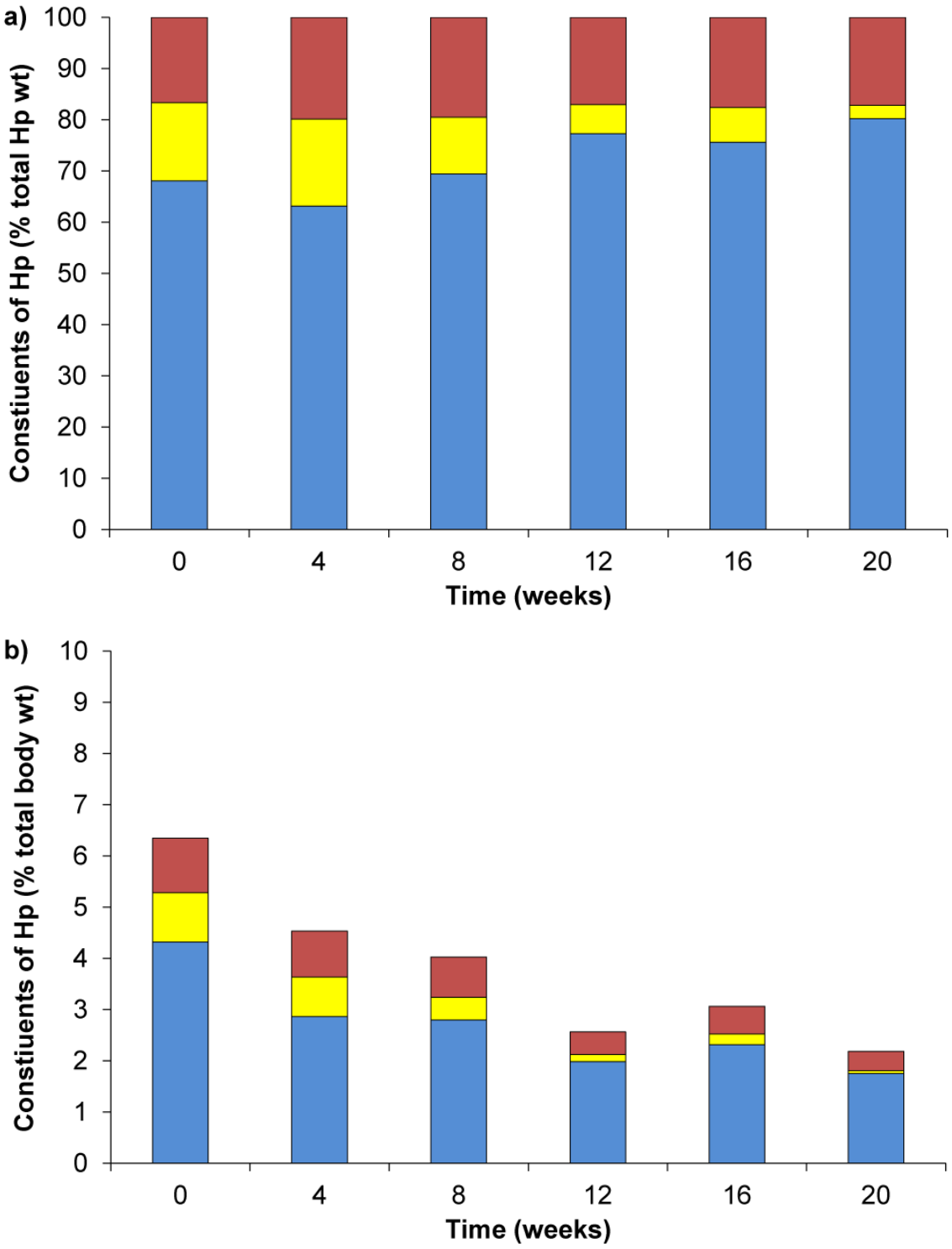

Figure 2.15 Changes in the constituent parts of the hepatopancreas during the starvation trial: a) proportional to total hepatopancreas weight, b) proportional to whole body weight. Colour code: mid-blue is hepatopancreas water; yellow is hepatopancreas lipids; maroon is everything else (including protein, glycogen and copper).n=51

3 Nutritional status in female *Nephrops norvegicus*: a comparison with male responses to starvation

3.1 Introduction

Chapter 2 established a methodology and time scale for assessing the nutritional status of male *Nephrops norvegicus*, and an aim of this chapter was to replicate this for females.

A major confounding factor when studying the biochemistry of female *N. norvegicus* is the large size variation of the female gonad, the ovary, which, when mature, can represent over 10% of the animal's body mass as explained in Chapter 1. The annual or biennial reproductive cycle in mature female *N. norvegicus* is another factor determining the nutritional status of the animal (Mente *et al.*, 2009). Large amounts of lipid, protein and carbohydrate are turned over during the developmental cycles of the female ovary, and Tuck *et al.* (1997a) suggested that these reserves would probably come from the hepatopancreas. However, no reduction in lipid, protein or carbohydrate was detected by them in the hepatopancreas during ovary maturation, as also reported by Rosa & Nunes (2002b). Both Tuck *et al.* (1997a) and Rosa & Nunes (2002b) suggest that females therefore consume more food prior to vitellogenesis, and it is for this reason that the turnover in lipids, protein and carbohydrate is seemingly maintained.

Due to this change in resource utilisation in females, some studies have used only males to assess the effect of starvation, e.g. those on *Cherax destructor* (Jones & Obst, 2000) and *Pandalus platyceros* (Whyte *et al.*, 1986). Parslow-Williams *et al.* (2001) examined RNA values in only male field-caught *N. norvegicus*, due to previously having found (Parslow-Williams, 1998) that there were significantly higher RNA values in tail muscle of males compared with those of females (Chapter 4 in Parslow-Williams, 1998). Other examples of physiological studies carried out solely on male crustaceans of various species are those on the carbohydrate mechanisms in the striped shore crab

Pachygrapsus crassipes (Schatzle *et al.*, 1973 and Dendinge & Schatzle, 1973), on seasonal variations in glycogen in the mid-gut of the blue crab *Callinectes sapidus* (Winget *et al.*, 1977), and on the effect of manganese on the chemically-mediated search behaviour of *N. norvegicus* (Krang & Rosenqvist, 2006). Other studies have avoided the physiological complications of the female reproductive cycle by using only juvenile animals, e.g. Hagerman (1983), Moss (1994), Rosa & Nunes (2003) and Pascual *et al.* (2006). Yet other studies have measured both males and females and commented on the differences. Thus Dall (1981) showed that male *N. norvegicus* have a lower lipid level in the hepatopancreas than females (15.38% and 13.12%, respectively), and Clarke (1979) showed that lipid levels in the hepatopancreas of male *Pandalus montugui* are between 1-2% of hepatopancreas weight, compared with a value of 3-4% in mature females.

The larger gonads found in females could provide additional resources such as lipids to starving animals (Rosa & Nunes, 2003). Therefore the various resources within the ovaries could potentially be used as starvation biomarkers for females. One major problem with using the resources of the ovaries in this way, however, is the difficulty of separating effects due to maturation cycles of the ovaries from the effects of the nutritional status of the female *per se*. For example, a fully mature ovary with a gonadosomatic index (GSI) of between 4-10% (Mente *et al.*, 2009) will have a larger amount of lipid than an immature ovary with a GSI of <1%.

Some studies, however, have not considered males and females separately, but rather have combined data from the two sexes. These include studies on commercially-caught *N. norvegicus,* where the relevant parameter is represented by the actually landed and marketed catch of mixed sexes (Thebault & Raffin, 1991; Albalat *et al.*, 2009). Other studies that give no indication of the sex of animals used are those of Dall (1975); Loo *et al.* (1993); Baden *et al.* (1990, 1994) and Stentiford *et al.* (2001a).

3.1.1 Aim

The main aims of the work described in this chapter (as in the previous chapter on males) were to describe the metabolic and physiological responses of female

N. norvegicus to starvation and to identify possible significant parameters that could be used to identify starvation in the field. The same three criteria as used in the previous chapter will be used, notably:

1. There is a clear difference between fed and unfed animals
2. This difference remains beyond a certain time point
3. It is insensitive to short term stresses caused by the method of collection (e.g. trawl capture).

To test what happens during post-capture recovery, animals will be compared at capture and after a two week acclimatisation time.

This chapter will also consider the effect of the subsequent feeding of females that were previously kept unfed, in an attempt to discriminate between the effects of long-term starvation and those of any subsequent short-term feeding.

3.2 Materials and Methods

3.2.1 Starvation trial

This aquarium trial was carried out with female *N. norvegicus*.

Trawled *N. norvegicus* were collected (on 11 Oct 2010) from the Clyde Sea Area north transect (Figure 1.5), UK (55°51.351′ N 4°54.424′ W to 55°48.979′ N 4°54.055′ W) by the RV Aora (UMBSM). Females with a mean (± SD) carapace length of 33.17 mm ± 5.11 were selected from the landed catch, a mixture of ovigerous and non-ovigerous females were used. These were then returned alive to the University of Glasgow on sea ice in sealed containers, and were held in an aquarium supplied with re-circulating filtered seawater for 2 weeks (with a 12h:12h light:dark photoperiod, at 9.4 °C ± 0.6 for the whole study) to allow them to recover from the stresses of capture and transport. Tanks were checked daily and any dead animals were removed.

The tanks contained short sections of upturned plastic guttering to provide refuges in which the females could reside (consistent with their burrow occupancy in the wild over this period). All animals were fed with *ca* 1g squid mantle three times a week (the ‘standard food ration’) for an initial 2-week

recovery period, until the trial started on 26 October 2010. Tanks were again checked daily and any dead animals were removed.

Animals were assigned randomly to one of 12 numbered tanks (n=62). The animals in two tanks had the standard food ration (fed group), food was removed from the tank within 20 hours of being placed in the tank, while those in the other ten tanks were starved (unfed group). One unfed animal was removed from each even-numbered tank at weeks 0, 8 and 16, and one unfed animal was removed from each odd-numbered tank at weeks 4, 12 and 20. As there were no major differences seen in the fed group in the trial with males (Chapter 2), the fed group of females was sampled only at the beginning of the trial and at weeks 12 and 20, to compare with the unfed group.

Animals were selected at random from each tank and put on ice for 10 minutes. Carapace length and weight without claws were measured. The hepatopancreas and ovary were then removed from the cephalothorax (noting the colour and weight of the organs), and the entire muscle was removed from the tail, prior to these three tissues being flash frozen in liquid nitrogen and then stored at -80°C prior to further analysis.

At the same time as the trial with females, a second aquarium trial with male *N. norvegicus* was also carried out, using animals that were collected and transported as described above (n=23). Four tanks were set up, two of which received the standard food ration while the other two did not. Groups of unfed animals were removed for analysis after each 4-week period as before (excluding week 16 due to a lack of animals). Fed animals were sampled only at week 12 and week 20.

3.2.2 Measurements

The water and lipid content and the concentrations of copper and glycogen in the hepatopancreas were measured. Determinations were also made of the water content and glycogen concentration in the abdominal tail muscle, and a stable isotope analysis of $\delta^{15}N$, $\delta^{13}C$ and the C:N ratio was also performed on this muscle. All protocols were the same as in described in Chapter 2, Section 2.2.

3.2.3 Subsequent feeding experiment

Twelve individuals from the unfed group of females were used in a 'subsequent feeding' trial. These animals were unfed for a total of 25 weeks prior to the start of this trial (21 April 2011). Six animals were fed again with the 'standard ration' that had been supplied to the fed group of animals, while six remained unfed. This feeding lasted for 4 weeks and all animals were then removed on week 29 (19 May 2011) alongside the group that had continually been unfed for 29 weeks. Both groups of animals were analysed for HSI, hepatopancreas constituents (water, lipids and copper) and tail constituents (water and lipids).

3.2.4 Stable isotope analysis of carapace

Stable isotope analyses were carried out on carapace (shell) selected from 3 animals from the unfed group at week 24 (12 April 2011) as well as on the carapaces of 3 field-caught animals from the Clyde Sea Area, (unknown month, 2009). The HSI was also measured in these animals to assess their nutritional status.

3.2.5 Statistical analysis

3.2.5.1 General Linear Models (GLMs)

Four separate statistical analyses (GLMs) were carried out for each measure. Due to the unbalanced nature of the data (there being a smaller number of fed animals than unfed animals) four tests were carried out:

Firstly, to assess the effect of the acclimatisation period (week -2 to week 0), individuals were also tested against each other to assess if the physiological measure had changed during the post-capture acclimatisation period. A significant difference between animals sampled at week -2 and at week 0 is displayed in each figure by a line over the two bars with an asterisk above it.

Secondly, to assess the effect of starvation period in all unfed animals between week 0 to week 20, a post hoc Tukey test was applied (when a significance was found) to assess the differences between means. These are displayed in the

figures as letters; means that do not share a letter are significantly different ($P<0.05$).

Thirdly and fourthly, the individuals sampled at week 12 and week 20 were separately tested for any significant difference between the fed and unfed groups, with week as a factor. As with the comparison between week -2 and week 0, significant differences will be indicated on the figures with a line and asterisk over the fed and unfed bars for each week.

Total number of observations in each table and figure where appropriate are reported as n=# or (when statistical output is present in tables) n=total df + 1, a breakdown of numbers of observations in each factor is displayed within the appendices.

3.3 Results

A total of 47 females were sampled throughout the 22 week trial. The mean sizes of the animals within the groups sampled at each 4-week time point did not vary significantly (all unfed ($F_{4,29}=1.37$, $P=0.273$), week 12 ($F_{1,7}=0.06$, $P=0.815$), week 20 ($F_{1,8}=1.93$, $P=0.207$)), indicating that there was no size bias in the sampling protocol (Figure 3.1).

Table 3.1 shows the gonad stage of each female used in the trial. This shows that, for the unfed group, beyond week 8 no ovary was at stage 3 (green) or above, and there was also a large proportion of animals with reabsorbed or spent ovaries. In this group, the GSI also decreased from around 2% at week -2 and 0 to around 0.5-1% from week 4 onward (Figure 3.2). However, fed individuals at both week 12 and at week 20 showed similarly reduced GSIs from the values at the start of the trial. Since there was no significant difference in the body size (measured as carapace length) between the groups of females at different time points, or between fed or unfed groups, it could be assumed that the mean GSI would also be similar between each group, if not being affected by another factor such as captive holding.

Table 3.1 The developmental stages of the gonads in both unfed and fed female *N. norvegicus* during the starvation trial, displayed as a) GSI; b) as gonad stages as described in Table 1.1. * indicates female was ovigerous.

	unfed						Fed					
a)	**1**	**2**	**3**	**4**	**5**	**6**	**1**	**2**	**3**	**4**	**5**	**6**
-2	-	-	-	-	-	-	0.87*	0.91*	0.74*	8.53	1.83	0.01
0	-	-	-	-	-	-	0.07	4.51	0.11	0.06	6.32	0.95
4	0.70	1.19	0.93	0.70	1.79	1.32	-	-	-	-	-	-
8	1.20	0.01	0.01	1.20*	0.01	0.01	-	-	-	-	-	-
12	1.17	1.78	1.73*	0.66	0.62	0.63	1.39	0.95*	-	-	-	-
16	1.01	0.55*	0.75	0.08	0.47*	0.50	-	-	-	-	-	-
20	1.39	1.69	1.43	0.09	0.98	1.47*	1.15	0.80*	0.10	-	-	-
b)	**1**	**2**	**3**	**4**	**5**	**6**	**1**	**2**	**3**	**4**	**5**	**6**
-2	-	-	-	-	-	-	1*	2*	1*	6	5	2
0	-	-	-	-	-	-	1	7	1	4	6	8
4	8	8	8	5	4	8	-	-	-	-	-	-
8	8	5	1	2*	8	8	-	-	-	-	-	-
12	8	3	4*	8	1	1	3	8*	-	-	-	-
16	8	1*	8	8	1*	1	-	-	-	-	-	-
20	8	8	3	1	2	4*	1	2*	1	-	-	-

3.3.1 Effects of withholding food in female *N. norvegicus*

The results of the four tests are shown in Table 3.2.

3.3.1.1 Metabolic depression - Phase 1

Copper

The mean values (± SE) of the copper concentration of the hepatopancreas of female *N. norvegicus*, between fed and unfed animals at each 4-week time point are shown in Figure 3.3.

There was no significant difference in the concentration of copper in the hepatopancreas between animals at the time of capture (139.48 ± 32.75 $\mu g.g^{-1}$) (11 Oct 2010) and after the two-week acclimatisation period at week 0 (181.96 ± 40.72 $\mu g.g^{-1}$) (26 Oct 2010) ($F_{1,11}$=0.66, P=0.435). Comparing unfed animals between sample points, there was an increase in the copper concentration of the hepatopancreas and this was bordering on significant ($F_{4,29}$=2.74, P=0.051). The copper concentration in the hepatopancreas of unfed animals increased to

371.64 ± 107.88 μg.g^{-1} by week 12, when it was higher than in animals that were fed, although this was not significant ($F_{1,7}$ =0.71, P=0.430). However, by week 20 this difference was bordering on significant at the 5% level ($F_{1,8}$ =5.48, P=0.052).

3.3.1.2 Reserve utilisation - Phase 2

Glycogen

The mean values (± SE) of the glycogen concentration of the a) hepatopancreas and b) abdominal tail muscle in female *N. norvegicus*, between fed and unfed animals at each 4-week time point are shown in Figure 3.4.

At capture the animals had a mean glycogen concentration in the hepatopancreas of 7.51 ± 3.91 mg.g^{-1}, and after 2 weeks acclimatisation this had not risen significantly (9.86 ± 3.14 mg.g^{-1}) ($F_{1,6}$=0.23, P=0.655). Comparing unfed animals between sample points, the animals at week 4 had a mean value of 4.93 ± 0.38 mg.g^{-1}, and this increased through the trial to become significantly higher by week 20, when it reached 17.94 ± 5.58 mg.g^{-1} ($F_{4,18}$=4.21, P=0.019).

Comparing fed and unfed groups, at week 12 there was a significant difference between them, with mean values of 22.77 ± 0.97 mg.g^{-1} for the fed group and 5.63 ± 1.79 mg.g^{-1} for the unfed group ($F_{1,7}$=26.84, P=0.002). However, by week 20 the mean value for the unfed group increased to 17.94 ± 5.58 mg.g^{-1}, and was then not significantly different from the mean value for the fed group (37.59 ± 27.86 mg.g^{-1}) ($F_{1,4}$=0.80, P=0.437), possibly due to the fact that the fed group at week 20 had a large SE (± 27.86).

At capture the animals had a mean glycogen concentration in the abdominal tail muscle of 0.49 ± 0.28 mg.g^{-1}, and after 2 weeks acclimatisation this had not risen significantly (3.04 ± 1.25 mg.g^{-1}) ($F_{1,11}$=3.98, P=0.074). There was no significant difference in this measure between weeks unfed ($F_{4,29}$=0.67, P=0.616) or between fed and unfed animals at week 12 ($F_{1,7}$=0.67, P=0.444) or at week 20 ($F_{1,8}$=0.99, P=0.354).

Lipid

The mean values (± SE) of the lipid content of the hepatopancreas in female *N. norvegicus*, between fed and unfed animals at each 4-week time point are shown in Figure 3.5.

There was a significant reduction in the amount of lipid in the hepatopancreas between animals at the time of capture (32.59 ± 2.43%) and after the two-week acclimatisation period (23.82 ± 0.81%) ($F_{1,7}$ =17.7, P=0.006). Thereafter, in the unfed group there was a reduction in the lipid content of the hepatopancreas over time ($F_{4,23}$=4.64, P=0.009). The post hoc Tukey test indicated that the mean value at week 20 (17.32 ± 4.61%) was significantly lower than those at weeks 8 (32.31 ± 1.60%), 12 (33.08 ± 2.59%) and 16 (31.09 ± 3.41%), whereas in the fed group there was no reduction. As a consequence, at week 20 there was a significant difference between the fed group (34.60 ± 1.51%) and unfed group (17.32 ± 4.61%) ($F_{1,7}$=7.67, P=0.032).

CL:weight ratio

The mean values (± SE) of the carapace length: weight ratio of female *N. norvegicus*, between fed and unfed animals at each 4-week time point are shown in Figure 3.6.

The CL: weight ratio in female *N. norvegicus* that were unfed did not vary significantly during the whole period of starvation ($F_{1,29}$=1.20, P=0.336). There was also no significant difference between fed and unfed groups at week 12 ($F_{1,7}$=0.57, P=0.478) or week 20 ($F_{1,8}$=2.05, P=0.196).

HSI

The mean values (± SE) of the hepatosomatic index (HSI) in female *N. norvegicus*, between fed and unfed animals at each 4-week time point are shown in Figure 3.7.

At capture the animals had a mean HSI of 5.88 ± 0.80% and after 2 weeks acclimatisation this had not changed significantly (7.24 ± 0.21%) ($F_{1,11}$=2.69, P=0.132). Thereafter there was no significant difference between the HSI values with time in the unfed group ($F_{1,29}$ =1.65, P=0.194). Comparing fed and unfed groups, there was no significant difference at week 12 between the fed group (7.79 ± 1.12%) and unfed group (6.13 ± 0.48%) ($F_{1,7}$= 2.67, P=0.153). However, by

week 20 the mean HSI value of unfed group had reduced to 4.96 ± 0.16%, and was then significantly lower than that of the fed group (6.23 ± 0.38%) ($F_{1,8}$=14.22, P=0.007).

Water content of tissues

The mean values (± SE) of the water content of the a) hepatopancreas and b) abdominal tail muscle in female *N. norvegicus*, between fed and unfed animals at each 4-week time point are shown in Figure 3.8.

There was no significant difference in the water content of the hepatopancreas between the animals at the point of capture (53.67 ± 3.31%) and after the 2-week acclimatisation period (56.96 ± 2.52%) ($F_{1,11}$=0.63, P=0.447). Through the trial period the water content increased in the hepatopancreas of the unfed group, the post hoc Tukey test revealing it to be significantly higher at week 20 (64.05 ± 3.22%) than at week 12 (51.94 ± 1.98%) ($F_{4,29}$=2.81, P=0.047). At week 12 there was no significant difference between the fed (52.48 ± 6.87%) and unfed groups (51.94 ± 1.98%) ($F_{1,7}$= 0.01, P=0.914). However, by week 20 the mean value for the unfed group (64.05 ± 3.22%) was significantly higher than that of the fed group (48.89 ± 1.09%) ($F_{1,8}$=10.13, P=0.015).

There was no significant difference in the water content of the abdominal tail muscle between the animals at the point of capture (76.84 ± 0.94%) and after the 2-week acclimatisation period (75.76 ± 0.45%) ($F_{1,11}$=1.07, P=0.326). There was also no significant difference among the unfed groups at different time points ($F_{4,29}$=1.06, P=0.397), or between the fed and unfed groups at week 12 ($F_{1,7}$=1.27, P=0.302) and week 20 ($F_{1,8}$=3.31, P=0.112).

Stable Isotopes

The mean values (± SE) of the stable isotopes of a) δ^{15}N and b) δ^{13}C in the abdominal tail muscle of female *N. norvegicus*, between fed and unfed animals at each 4-week time point are shown in Figure 3.9.

There was no significant difference in the δ^{15}N value of the abdominal tail muscle between the animals at the point of capture (13.08 ± 0.16‰) and after the 2-week acclimatisation period (13.47 ± 0.16‰) ($F_{1,11}$=2.85, P=0.123).There was also no significant difference among unfed groups at different time points

($F_{4,29}$=0.57, P=0.688), or between fed and unfed groups at week 12 ($F_{1,7}$=3.11, P=0.128) and week 20 ($F_{1,8}$=1.38, P=0.279).

There was also no significant difference in the δ^{13}C value of the abdominal tail muscle between the animals at the point of capture (-15.59 ± 0.17‰) and after the 2-week acclimatisation period (-15.66 ± 0.09‰) ($F_{1,11}$=0.15, P=0.708). There was also no significant difference among unfed groups at different time points ($F_{4,29}$=1.48, P=0.239), or between fed and unfed groups at week 12 ($F_{1,7}$=0.04, P=0.846) and week 20 ($F_{1,8}$=1.63, P=0.243).

The mean values (± SE) of the C:N ratio of the abdominal tail muscle of female *N. norvegicus*, between fed and unfed animals at each 4-week time point are shown in Figure 3.10.

There was no significant difference in the C:N ratio of the abdominal tail muscle between the animals at the point of capture (3.12 ± 0.02) and after the 2-week acclimatisation period (3.11 ± 0.03) ($F_{1,11}$=0.10, P=0.753).There was also no significant difference among unfed groups at different time points ($F_{4,29}$=0.44, P=0.778), or between fed and unfed groups at week 12 ($F_{1,7}$=0.39, P=0.557) and week 20 ($F_{1,8}$=1.02, P=0.347).

3.3.1.3 Change in hepatopancreas composition

The mean values of each constituent of the hepatopancreas at each time point in the trial expressed as a percentage, by weight, of the hepatopancreas are shown in Figure 3.11.

The first graph shows that, as with males (Chapter 2), the percentage of lipids and water contained in the hepatopancreas accounts for *ca* 80% of the total weight of the hepatopancreas. The mean value of the sum of these two measures, combining data for the fed or unfed groups, was 83.55 ± 0.64%. There was no significant difference in the combined percentage of the hepatopancreas made up of water and lipid, in any unfed group ($F_{4,23}$=0.37, P=0.830).

3.3.1.4 Carapace SIA

As no significant difference was observed in the stable isotope values of $\delta^{15}N$ and $\delta^{13}C$ in the tail muscle of female *N. norvegicus* after 20 weeks of starvation (Figure 3.9), measurements were made on the carapaces of three animals (at week 24 of the trial), to determine if any changes could be detected in this structural tissue after this period of starvation. The carapace $\delta^{15}N$ values in these unfed individuals were significantly lower compared to the values found in animals taken directly from the field ($F_{1,5}$ =13.66, P=0.021) (Table 3.3). The HSI values obtained from the unfed animals were also significantly lower than the animals taken directly from the field ($F_{1,5}$ =12.98, P=0.023), confirming a reduced nutritional status in the trial animals.

Table 3.2 General Linear Model applied to measures made on females in the starvation trial. a) The effect of the acclimatisation period (weeks -2 and 0). b) The temporal effect of starvation: a post hoc Tukey test was applied (when a significance was found) to assess the differences between means. c) The difference between fed and unfed females at week 12. d) The difference between fed and unfed females at week 20. n= total df + 1

a)	**between week -2 and 0**				**means**	
	F	**df**	**P**	**r^2 (%)**	**-2**	**0**
Carapace length (mm)	0.77	1,11	0.402	7.11	29.48 ± 2.69	32.53 ± 2.22
Hepatopancreas copper (µg.g^{-1} wet)	0.66	1,11	0.435	6.20	139.48 ± 32.75	181.96 ± 40.72
Hepatopancreas glycogen (mg.g^{-1} wet)	0.23	1,6	0.655	4.31	7.51 ± 3.91	9.86 ± 3.14
Tail muscle glycogen (mg.g^{-1} wet)	3.98	1,11	0.074	28.47	0.49 ± 0.28	3.04 ± 1.25
Hepatopancreas lipid (% wet wt)	17.74	1,7	0.006	74.73	32.59 ± 2.43	23.82 ± 0.81
CL: weight ratio	0.42	1,11	0.532	4.02	1705.04 ± 233.63	1857.62 ± 32.65
HSI (% wet wt)	2.69	1,11	0.132	21.17	5.88 ± 0.80	7.24 ± 0.21
Hepatopancreas water (%)	0.63	1,11	0.447	5.88	53.67 ± 3.31	56.96 ± 2.52
Tail muscle water (%)	1.07	1,11	0.326	9.64	76.84 ± 0.94	75.76 ± 0.45
Tail muscle $\delta^{15}N$ (‰)	2.85	1,11	0.123	22.15	13.08 ± 0.16	13.47 ± 0.16
Tail muscle $\delta^{13}C$ (‰)	0.15	1,11	0.708	1.46	-15.59 ± 0.17	-15.66 ± 0.09
Tail muscle C: N ratio (wt)	0.10	1,11	0.753	1.03	3.12 ± 0.02	3.11 ± 0.03

Table 3.2 continued

b) n=30	between all unfed				means and Tukey				
	F	**df**	**P**	**r^2**	**4**	**8**	**12**	**16**	**20**
Carapace length (mm)	1.37	4,29	0.273	17.98	36.32 ± 2.75	32.35 ± 2.06	38.43 ± 3.32	32.42 ± 2.21	39.25 ± 3.33
Hepatopancreas copper (µg.g^{-1} wet)	2.74	4,29	0.051	30.51	245.97 ± 33.49	226.76 ± 42.04	371.64 ± 107.88	373.19 ± 74.92	531.87 ± 85.07
Hepatopancreas glycogen (mg.g^{-1} wet)	4.21	4,18	**0.019**	54.58	4.93 ± 0.38 **b**	4.47 ± 1.62 **b**	5.63 ± 1.79 **b**	6.54 ± 1.94 **ab**	17.94 ± 5.58 **a**
Tail muscle glycogen (mg.g^{-1} wet)	0.67	4,29	0.616	9.75	2.79 ± 1.35	0.90 ± 0.43	2.30 ± 0.91	2.32 ± 1.04	2.90 ± 0.90
Hepatopancreas lipid (% wt)	4.64	4,23	**0.009**	49.39	28.95 ± 2.25 **ab**	32.31 ± 1.60 **a**	33.08 ± 2.59 **a**	31.09 ± 3.41 **a**	17.32 ± 4.61 **b**
CL: weight ratio	1.20	4,29	0.336	16.10	1771.95 ± 75.45	1737.80 ± 65.48	1872.40 ± 47.73	1729.28 ± 74.63	1868.17 ± 49.56
HSI (% wet wt)	1.65	4,29	0.194	20.84	5.37 ± 0.53	5.38 ± 0.46	6.13 ± 0.48	4.72 ± 0.35	4.96 ± 0.16
Hepatopancreas water (%)	2.81	4,29	**0.047**	31.02	55.14 ± 1.43 **ab**	55.77 ± 3.43 **ab**	51.94 ± 1.98 **b**	56.73 ± 2.74 **ab**	64.05 ± 3.22 **a**
Tail muscle water (%)	1.06	4,29	0.397	14.50	75.23 ± 0.23	75.93 ± 0.72	75.12 ± 0.43	76.13 ± 0.40	76.09 ± 0.44
Tail muscle δ^{15}N (‰)	0.57	4,29	0.688	8.34	13.53 ± 0.18	13.58 ± 0.17	13.74 ± 0.15	13.70 ± 0.12	13.35 ± 0.33
Tail muscle δ^{13}C (‰)	1.48	4,29	0.239	19.10	-15.36 ± 0.11	-15.36 ± 0.09	-15.47 ± 0.12	-15.58 ± 0.13	-15.09 ± 0.24
Tail muscle C: N ratio (wt)	0.44	4,29	0.778	6.59	3.07 ± 0.02	3.06 ± 0.03	3.10 ± 0.03	3.05 ± 0.02	3.05 ± 0.05

Table 3.2 continued

c)	week 12 fed and unfed				means	
	F	**df**	**P**	**r^2**	**fed**	**unfed**
Carapace length (mm)	0.06	1,7	0.815	0.98	36.90 ± 3.60	38.43 ± 3.32
Hepatopancreas copper ($\mu g.g^{-1}$ wet)	0.71	1,7	0.430	10.64	204.59 ± 35.09	371.64 ± 107.88
Hepatopancreas glycogen ($mg.g^{-1}$ wet)	26.84	1,7	**0.002**	81.73	22.77 ± 0.97	5.63 ± 1.79
Tail muscle glycogen ($mg.g^{-1}$ wet)	0.67	1,7	0.444	10.06	3.67 ± 0.39	2.30 ± 0.91
Hepatopancreas lipid (% wt)	0.38	1,6	0.567	6.99	30.37 ± 1.68	33.08 ± 2.59
CL: weight Ratio	0.57	1,7	0.478	8.72	1804.40 ± 46.02	1872.40 ± 47.73
HSI (% wet wt)	2.67	1,7	0.153	30.80	7.79 ± 1.12	6.13 ± 0.48
Hepatopancreas water (%)	0.01	1,7	0.914	0.21	52.48 ± 6.87	51.94 ± 1.98
Tail muscle water (%)	1.27	1,7	0.302	17.50	74.21 ± 0.44	75.12 ± 0.43
Tail muscle $\delta^{15}N$ (‰)	3.11	1,7	0.128	34.14	14.22 ± 0.11	13.74 ± 0.15
Tail muscle $\delta^{13}C$ (‰)	0.04	1,7	0.846	0.68	-15.52 ± 0.08	-15.47 ± 0.12
Tail muscle C: N ratio (wt)	0.39	1,7	0.557	6.05	3.14 ± 0.01	3.10 ± 0.03

Table 3.2 continued

d)	week 20 fed and unfed				Means (± SE)	
	F	**df**	**P**	**r^2**	**fed**	**unfed**
Carapace length (mm)	1.93	1,8	0.207	21.61	31.97 ± 2.97	39.25 ± 3.33
Hepatopancreas copper ($\mu g.g^{-1}$ wet)	5.48	1,8	0.052	43.93	234.70 ± 37.22	531.87 ± 85.07
Hepatopancreas glycogen ($mg.g^{-1}$ wet)	0.80	1,4	0.437	21.03	37.59 ± 27.86	17.94 ± 5.58
Tail muscle glycogen ($mg.g^{-1}$ wet)	0.99	1,8	0.354	12.34	1.45 ± 0.94	2.90 ± 0.90
Hepatopancreas lipid (% wt)	7.67	1,7	**0.032**	56.10	34.60 ± 1.51	17.32 ± 4.61
CL: weight ratio	2.05	1,8	0.196	22.61	1726.88 ±102.72	1868.17 ± 49.56
HSI (% wet wt)	14.22	1,8	**0.007**	67.02	6.23 ± 0.38	4.96 ± 0.16
Hepatopancreas water (%)	10.13	1,8	**0.015**	59.15	48.89 ± 1.09	64.05 ± 3.22
Tail muscle water (%)	3.31	1,8	0.112	32.11	74.74 ± 0.57	76.09 ± 0.44
Tail muscle $\delta^{15}N$ (‰)	1.38	1,8	0.279	16.44	13.95 ± 0.24	13.35 ± 0.33
Tail muscle $\delta^{13}C$ (‰)	1.63	1,8	0.243	18.86	-15.57 ± 0.17	-15.09 ± 0.24
Tail muscle C: N ratio (wt)	1.02	1,8	0.347	12.69	3.12 ± 0.04	3.05 ± 0.05

Table 3.3 GLM results from the trial comparing various measurements on the carapace in field caught and unfed female *N. norvegicus*. Bold P values indicate significance (P<0.05). Means and standard error values are displayed. n= total df + 1

	Between field and unfed				Means (± SE)	
	F	**df**	**P**	**r^2**	**field**	**unfed**
Carapace length (mm)	3.00	1,5	0.158	42.83	32.90 ± 0.59	31.37 ± 0.66
HSI (% wet wt)	12.98	1,5	**0.023**	76.44	7.58 ± 0.55	5.35 ± 0.29
Carapace δ^{15}N (‰)	13.66	1,5	**0.021**	77.35	8.80 ± 0.20	7.48 ± 0.30
Carapace δ^{13}C (‰)	0.18	1,5	0.690	4.41	-14.47 ± 0.55	-14.20 ± 0.28
Carapace C:N ratio (wt)	0.34	1,5	0.592	7.80	5.15 ± 0.42	5.41 ± 0.16

3.3.2 Effects of reproductive condition

To assess the effect of egg presence on the mean values of the measured parameters, the individual values of measurements of ovigerous females in comparison to the mean value of that measure for all females at each sampled time point are shown in Table 3.4. There were 10 ovigerous females out of the total of 47 sampled during the trial. This indicates that when female *N. norvegicus* are ovigerous the lipid content of the hepatopancreas was slightly higher and the water content lower than in non-ovigerous females. Also the copper concentration of the hepatopancreas was lower in ovigerous females. These observations are not statistically rigorous; however they do provide an indication of the difference that may exist between reproductive stages.

Table 3.4 Mean values (± SE) of HSI, hepatopancreas water, copper and lipid for groups of all fed and unfed females at time points from -2 weeks to 20 weeks. Red values indicate ovigerous females. Females -2c-e and 20c had light green eggs; females 8a, 12a 12c, 16a-b, 20a had dark green eggs.

HSI

	Not fed				Fed				
	Mean	SE	a	b	Mean	SE	c	d	e
-2					5.88	0.08	7.83	2.28	6.61
0					7.24	0.21			
4	5.37	0.53							
8	5.38	0.46	5.30						
12	6.13	0.48	7.51		7.79	1.12	6.67		
16	4.72	0.35	5.46	5.16					
20	4.96	0.16	4.76		6.23	0.37	5.62		

Hepatopancreas water

	Not fed				Fed				
	Meam	SE	a	b	Mean	SE	c	d	e
-2					53.67	3.31	47.10	69.05	51.69
0					56.96	2.52			
4	55.14	1.43							
8	55.77	3.43	49.29						
12	51.94	1.98	44.76		52.48	6.87	59.36		
16	56.73	2.74	46.60	54.88					
20	64.05	3.22	60.28		48.89	1.09	48.55		

Hepatopancreas copper

	Not fed				Fed				
	Mean	SE	a	b	Mean	SE	c	d	e
-2					139.48	32.75	62.39	264.89	172.58
0					181.96	40.72			
4	245.97	33.49							
8	226.76	42.04	122.37						
12	371.64	107.88	114.77		204.59	35.09	169.50		
16	373.19	74.92	292.25	145.39					
20	531.87	85.07	339.28		234.70	37.22	321.51		

Hepatopancreas lipid

	Not fed				Fed				
	Mean	SE	a	b	Mean	SE	c	d	e
-2					32.59	2.43	27.85		35.92
0					23.82	0.81			
4	28.95	2.25							
8	32.31	1.60	34.23						
12	33.08	2.59	38.49		30.37	1.68	28.69		
16	31.09	3.41	38.51	22.05					
20	17.32	4.61	22.79		34.60	1.51	32.76		

3.3.3 Subsequent feeding trial

The mean values (± SE) for HSI, water content of the tail, lipid and water content and copper concentration of the hepatopancreas, in female *N. norvegicus* that had previously been starved for 25 weeks and then subsequently fed for 4 weeks on the standard ration have been compared with the group that continued to have food withheld (Figure 3.12).

There were slight differences between the means of each measure indicating a possible increase in nutritional status. However, none of these differences was significant (Table 3.5).

Table 3.5 GLM output from the subsequent feeding trial comparing various measurements in the unfed and subsequently fed group for 4 weeks. Means and standard error values are presented. n= total df + 1

	F	df	P	r^2 (%)	Starved	Subsequent feeding
HSI (% wet wt)	4.37	1,11	0.063	30.43	3.93 ± 0.55	5.16 ± 0.21
Hepatopancreas water (% wt)	1.51	1,11	0.247	13.14	71.78 ± 2.95	67.39 ± 2.01
Tail muscle water (% wt)	1.79	1,11	0.211	15.18	76.87 ± 0.36	76.22 ± 0.33
Hepatopancreas lipid (% wet wt)	2.17	1,8	0.171	17.84	37.70 ± 9.70	40.92 ± 3.08
Hepatopancreas copper ($\mu g.g^{-1}$ wet)	0.02	1,9	0.897	0.22	673.74 ± 167.87	700.31 ± 119.42

3.3.4 Comparison with males

The responses of females to starvation can be compared with the responses of both the males from the first trial (held at the higher temperature of 11.7°C) (Chapter 2) and with those males held at the same temperature as the females (9.4°C) over the same trial period. The responses of unfed groups of these males and females were compared at weeks 12 and 20 (Table 3.6).

HSI

The mean HSI values of two male unfed groups and the female unfed group at week 12 varied significantly ($F_{2,13}$=24.90, P<0.001). The females had the highest mean value (6.13 ± 0.48%), which was significantly higher than that of males held at the higher temperature in the first trial (2.56 ± 0.23%). The males in the second trial that were held at the same temperature as the females had a mean HSI value (4.28 ± 0.11%) that was not significantly different from either the males in the first trial or the females (Tukey, P<0.05).

The mean HSI values of two male unfed groups and the female unfed group at week 20 also varied significantly ($F_{2,11}$=20.57, P<0.001). The mean HSI value of males from the second trial (3.08 ± 0.47%) was significantly lower than that of the females (4.96 ± 0.16%), but still not significantly different from that of the males in the first trial (2.19 ± 0.5%) (Tukey, P<0.05).

Water

The water content of the hepatopancreas of the three unfed groups sampled at week 12 varied significantly ($F_{2,13}$=56.86, P<0.001), being lowest in the females (51.94 ± 1.98%), compared with the males from the second trial (63.99 ± 2.89%) and the males from the first, higher temperature trial (77.28 ± 1.32%) (Tukey, P<0.05). By week 20 the water content of the hepatopancreas of females had increased, and was significantly higher than males from the first trial to ($F_{2,11}$=7.55, P=0.012), but no longer significantly different from males in the second trial (Tukey, P<0.05).

Lipid

The lipid content of the hepatopancreas of the three unfed groups sampled at week 12 varied significantly ($F_{2,9}$=26.15, P=0.001). From the second trial the values for the females (33.08 ± 2.59%) and the males (24.90 ± 3.99%) were not significantly different from each other, but both were significantly higher than the value for the males in the first trial (5.63 ± 2.02%) (Tukey, P<0.05). By week 20 there were no longer any significant differences between the three groups ($F_{2,9}$=4.07, P=0.067), due to substantial falls in the values for the males and females in the second trial (Table 3.6).

Copper

The copper concentration of the hepatopancreas of the three unfed groups sampled at week 12 varied significantly ($F_{2,11}$=5.00, P=0.035), with males from the first trial having a significantly higher mean value (635.06 ± 135.58 $\mu g.g^{-1}$) than males from the second trial (99.65 ± 24.83 $\mu g.g^{-1}$), although not significantly higher than the females (371.64 ± 107.88 $\mu g.g^{-1}$) (Tukey, P<0.05). By week 20 the copper concentrations in the two groups of the second trial had increased substantially, so that there were then no significant differences among the three groups ($F_{2,9}$=0.42, P=0.673).

Table 3.6 GLM output of the comparison between all three trial groups: the male group from the first trial (held at 11.7°C) and the male and female groups from the second trial (held at 9.4°C). Means and standard errors presented. Means that do not share a letter are significantly different. n= total df + 1

Week 12	F	df	P	r^2 (%)	1st male trial	2nd male trial	Female trail
HSI	24.90	2,13	**<0.001**	81.91	2.56 ± 0.23 **b**	4.28 ± 0.11 **ab**	6.13 ± 0.48 **a**
Water	56.86	2,13	**<0.001**	91.18	77.28 ± 1.32 **a**	63.99 ± 2.89 **b**	51.94 ± 1.98 **c**
Lipid	26.15	2,9	**0.001**	88.2	5.63 ± 2.02 **b**	24.90 ± 3.99 **a**	33.08 ± 2.59 **a**
Copper[1]	5.00	2,11	**0.035**	52.64	635.06 ± 135.58 **a**	99.65 ± 24.83 **b**	371.64 ± 107.88 **ab**
				log copper	2.77 ± 0.10 **a**	1.99 ± 0.11 **b**	2.46 ± 0.14 **ab**

Week 20	F	df	P	r^2 (%)	1st male experiment	2nd male experiment	Female experiment
HSI	20.57	2,11	**<0.001**	82.05	2.19 ± 0.5 **b**	3.08 ± 0.47 **b**	4.96 ± 0.16 **a**
Water	7.55	2,11	**0.012**	62.64	80.21 ± 1.56 **a**	70.60 ± 4.13 **ab**	64.05 ± 3.22 **b**
Lipid	4.07	2,9	0.067	53.77	2.58 ± 1.32	4.55	17.32 ± 4.61
Copper	0.42	2,9	0.673	10.68	338.16 ± 15.93	646.16 ± 428.98	531.87 ± 85.07

[1] log base 10 transformed to meet normality

3.4 Discussion

The range of internal mechanisms which female *Nephrops norvegicus* employ to survive extended periods of starvation (20 weeks) are in the main consistent with those observed in males (Chapter 2), with some notable exceptions, which will be described below.

3.4.1 Effects of food deprivation on females compared to males

Firstly, and most importantly, the timings of the effects were different. While for the males (in the first trial) significant differences between fed and unfed groups were apparent at week 12 in the copper, lipid and water content of the hepatopancreas, these differences did not become significant in females until week 20.

There was no significant effect of starvation on any of the measures made on the tail muscle within the time scale of the female trial. A slightly lower C:N ratio was observed in unfed animals compared with fed, but the difference was not significant at either week 12 or week 20. Males started with a higher C:N ratio (3.16) compared with females (3.11). Over the trial period this declined in both sexes, but to a greater extent in males (to 2.99) than in females (to 3.05), so that by the end of the trial it was in fact lower in males than in females. If the C:N ratio is taken as a proxy for lipid content as proposed for Collembola by Haubert *et al.* (2005) and Semenina & Tiunov (2011), then this suggests that females have less tail muscle lipid initially. However, tail muscle lipids were not measured in females, so this cannot be confirmed at this stage.

There was no significant difference in the stable isotope values of tail muscle between samples of unfed and fed female *N. norvegicus*. At the end of the trial, however, the fed animals had larger $\delta^{15}N$ values than the animals at the start. This is consistent with the theory that they are equilibrating to the food source provided in the trial (in this case squid mantle, which had a $\delta^{15}N$ value of *ca* 14‰), and consistent with the results observed in the male experiment.

There was a significant difference in the $\delta^{15}N$ values in the carapace, with higher values in the field-caught samples compared with the aquarium-held, unfed animals. The HSI was used as an independent measure to confirm that the aquarium-held animals were in a starved state and the field-caught animals were not. Taken together including HSI, these findings indicate that $\delta^{15}N$ in the carapace actually declined in starved animals, which is opposite to what has been found in various soft tissues after similar trials (Haubert *et al.*, 2005; Semenina & Tiunov, 2011). These preliminary results cannot exclude the possibility of a temporal variation of $\delta^{15}N$ in the field to explain the observed variation in the carapace $\delta^{15}N$. A controlled aquarium-based trial is required in order to fully evaluate whether these changes are indeed related to starvation, and how nitrogen sources are used to maintain the structure of the carapace.

Lipid levels in the hepatopancreas of female *N. norvegicus* decreased with starvation. This reserve, however, was maintained at a higher level (17.32 ± 4.61% of the total tissue weight) than in males (2.58 ± 1.31%) after 20 weeks of starvation. This finding is consistent with Clarke (1979) who showed that the male shrimp *Pandalus montagui* had hepatocreatic lipid levels of 1-2%, while in mature females they were maintained at around 3-4%. He also stated that immature females have lipid values that are consistent with male values, indicating that the observed differences between mature males and females relate to the presence of developed gonads. Tuck *et al.* (1997a) and Rosa & Nunes (2002b) suggested that at the later stages of ovary development, females would have to consume more food in order to acquire sufficient cholesterol to produce steroid hormones for ovary maturation, and to build up more lipid reserves prior to returning to their burrows for egg incubation.

The lipid and water content in the female hepatopancreas also showed an inverse relationship, with a significant correlation comparable to that found in males. This finding is consistent with Hardy *et al.* (2000) who showed a significant correlation between water and lipid in the hepatopancreas of *Chionoecetus opilio*.

The copper concentration in the hepatopancreas of unfed females at week 12 was 371.64 ± 107.88 $\mu g.g^{-1}$ and increased at week 20 to 531.87 ± 85.07 $\mu g.g^{-1}$. By

week 29 (the end of the 'subsequent feeding' trial) those animals that had remained unfed had a hepatopancreas copper concentration further increased to 673.74 ± 167.8 $\mu g.g^{-1}$. For comparison, the highest mean value of copper in the hepatopancreas of unfed males was recorded in the group which was unfed for 16 weeks, when it had a value of 456.50 ± 158.88 $\mu g.g^{-1}$.

Baden *et al.* (1994) showed that the copper concentration of a group of mixed sex *N. norvegicus* that had been starved for 7 months reached a value of 1,550 ± 254 $\mu g.g^{-1}$, expressed as dry weight. If the values obtained in the present study are similarly expressed as dry weights, then the results for the unfed group of females are equivalent at week 20 (1,632.57 ± 390.45 $\mu g.g^{-1}$ hepatopancreas copper) to those obtained by Baden *et al.* (1994). However, applying the same conversion to the equivalent data from males yields values that are much larger (hepatopancreas copper 2976.01 ± 660.86 $\mu g.g^{-1}$ at 20 weeks unfed). This difference is due to the fact that the water content of the hepatopancreas of the male is greater than that of the female (80.21 ± 1.56% and 64.05 ± 3.22%, respectively).

The glycogen concentration in the hepatopancreas of female *N. norvegicus* in this study was very low at the beginning of the trial for both fed and unfed individuals, and did not change significantly during the initial two-week acclimatisation period in either the hepatopancreas or the tail muscle. Females therefore seem not to recover as quickly from the exhaustive stresses of trawling (Figure 2.4), in comparison with males. Subsequently, however, by week 20 there were signs of recovery in unfed female *N. norvegicus*.

The HSI also decreased in unfed female *N. norvegicus* with time, and the difference in HSI between fed and unfed animals was significant at week 20 in females (mean 4.96 ± 0.16%). There is, however, no significant difference between all the unfed animal data when comparing each time point. Figure 3.11b shows that the HSI in unfed animals did not decrease from the start to the end of the experiment and week 20 fed individuals could be significantly higher than all the unfed animals. HSI values were also much higher than those of males. The lowest value of HSI in females was 2.30%, this being much higher than the lowest value found in males (1.46%).

The hepatopancreas water content in females (55.14 - 64.05%) was much lower than that in males (68.05 - 80.21%). Conversely, the lipid content of the hepatopancreas in females (28.94 - 17.32%) was much higher than in males (15.22 - 2.58% over the two trials). The elevated lipid reserves in the females contribute to their higher HSI.

3.4.2 Subsequent feeding

A recovery (feeding) stage is an important consideration when assessing measurements of starvation. If an animal was caught that has not been feeding for 6 months whilst in the burrow but feeds for 2 days before being captured, it is necessary to be confident that any measure of nutritional status reflects the 6 months of starvation and not the 2 days of feeding. As subsequent feeding at week 25 for four weeks did not produce any significant difference from animals left without food, it could be assumed that these measurements are valid for at least four weeks when *N. norvegicus* is not fed for this period of time. Zhang *et al.* (2009) have demonstrated that adaptation to starvation conditions is a common feature in crustacean species. They also suggested that animals have a point-of-no-return (PNR) after which they are not be able to recover. It is unclear from the present study if *N. norvegicus* which were unfed for 25 weeks were taken beyond their PNR, since re-feeding for 4 weeks induced no significant reversal in the measured parameters. In a future study it would be useful to establish when the PNR would be reached in *N. norvegicus*. For periods without food shorter than the PNR, it would also be instructive to determine the time taken for subsequent feeding to re-establish normal levels of metabolic activity and nutrient reserves.

3.4.3 Effects of reproductive condition

The effect of the ovigerous condition on the responses of females to starvation was assessed by considering separately the females in the trial that were bearing eggs. The decrease in lipid and increase in water content of the hepatopancreas during starvation seemed to be less marked in ovigerous females, compared with non-ovigerous females (although it was not possible to test this statistically). Also, the hepatopancreas copper was lower and lipid higher in unfed ovigerous

females, suggesting that even after periods of starvation the internal reserves measured are maintained.

One reason for these reserves being maintained was suggested by Tuck *et al.* (1997a) and Rosa & Nunes (2003). The gonads contain many biochemical resources that could be utilised for energy, leading to their reabsorption, and indeed in non-ovigerous females in this study the ovaries seemed to be reabsorbed throughout the trial. Beyond week 8 there was no ovary at stage 3 (green) or subsequent stages. This breakdown (or reabsorption) of the ovaries could be providing resources and thus may be one of the reasons why females appear to be at a less starved level than males. Tuck *et al.* (1997a) suggested that reabsorption of the ovary is due to lack of fertilisation, starvation, hormone deprivation or incorrect photoperiod. As reabsorption occurred in both fed and unfed groups, the photoperiod and /or lack of fertilisation may also have been factors. However, unfed animals reabsorbed more readily (Figure 3.2) suggesting that the combined effects of photoperiod, lack of fertilisation and starvation leads to females abandoning their normal annual reproductive cycle.

3.4.4 Temperature

Variation in temperature has a universal effect on the physiological processes in all organisms (Lagerspetz & Vainio, 2006). This is especially the case for ectothermic organisms where external temperature exerts a major influence on the metabolic function of animals (Hawkins, 1995; Angilletta *et al.*, 2002), and certainly would have been an important factor in the present work. Hardy *et al.* (2000) showed that there was a significant difference in mortality, gut weight, and the water and lipid content of the digestive gland (hepatopancreas) of the snow crab *Chionoecetes opilio* after a starvation period of 154 days at three different temperatures (1˚C, 5˚C, 10˚C). The results presented here are consistent with those of their study. Worden *et al.* (2006) showed that the heart rate of *Homarus americanus* is much lower at temperatures around 2˚C (*ca* 20 beats min^{-1}), rising to 40 bpm at 10˚C and then declining again to 10 bpm at 22˚C. Therefore the rate of utilisation of a reserve is a function of the temperature at which the animal is held. For example, an animal held at a lower temperature will use fewer lipid reserves under a starved condition. This is

known as the temperature-dependent rate process, where at low temperatures there is inactivity and at medium temperature there is increased activity (Lagerspetz & Vainio, 2006). These temperature-dependent responses induced by holding lobsters at *ca* 5°C, are utilised by the companies distributing live lobsters in order to keep them in a good condition prior to shipping. In Chapter 1 it was noted that metabolic depression occurs intrinsically (MD_i) due to starvation, but is also temperature dependent (MD_t) (McCue, 2010).

In the present study, the results from female *N. norvegicus* suggest that in response to food withdrawal the metabolic depression phase was prolonged, meaning that less utilisation of biochemical resources took place, resulting in more resources remaining than in their male counterparts in the first trial. It is not clear whether this was a sex effect (MD_i) or a temperature effect (MD_t). At week 12, in comparison with males at the same temperature, water and lipids were still indicating less nutritional stress in females, with more lipid reserves and less water. However, beyond week 12 the males and females studied at the same temperature had no significant difference in HSI, lipid content or copper concentration of the hepatopancreas. The HSI and lipid were significantly different between the male *N. norvegicus* in the two different temperature experiments. By week 20 the copper concentration of the hepatopancreas in males and females held at the same temperature were still not significantly different from each other and the difference in the water content of the hepatopancreas became non-significant. This suggests that in the second starvation trial the metabolic depression phase involved a combination of MD_i and MD_t. Therefore any measure in the field must be interpreted in terms of the prevailing ambient water temperature.

It cannot be known what the outcome of the first trial would have been if it had involved both females and males, compared at the higher temperature (11.7˚C). However, as both sexes seemed to react similarly in the second trial (at 9.4˚C), in terms of water and copper increasing and lipids decreasing in the hepatopancreas, it is concluded that the responses of the two sexes to starvation are essentially the same.

3.4.5 Measures that could be used to indicate starvation of females in the field

Of the parameters used in the starvation trials on females, a number satisfy the three criteria for use as potential biomarkers of this condition in field sampled animals as shown in Section 3.1.1.

As Table 3.2 indicates, as with males (Chapter 2), the hepatopancreas copper, lipid and water and the HSI, could be used in this way.

As glycogen in the hepatopancreas and the muscle is known to vary between individual *N. norvegicus* for a number of reasons (e.g. activity levels, moulting condition, reproductive cycle), and is also affected significantly by the trawl capture process used to obtain the samples (Albalat *et al*., 2009), it fails to meet all the criteria for being a suitable biomarker.

There was no significant effect of starvation on any of the tail muscle measures of females within the time scales considered, so none of these offer potential biomarkers.

The validation of starvation biomarkers for male and female *N. norvegicus* is considered in detail in the following chapter (Chapter 4).

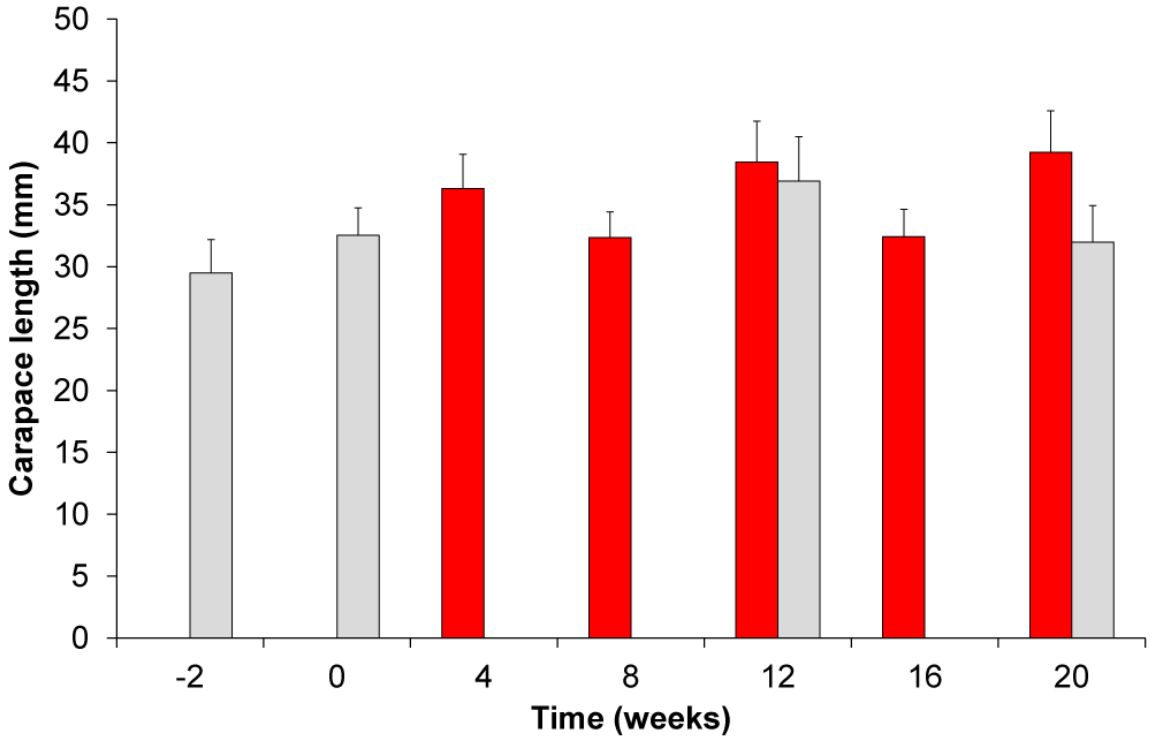

Figure 3.1 Carapace length (means ± SE) of unfed (red bars) and fed (grey bars) groups of female *N. norvegicus* during the starvation trial. n=47

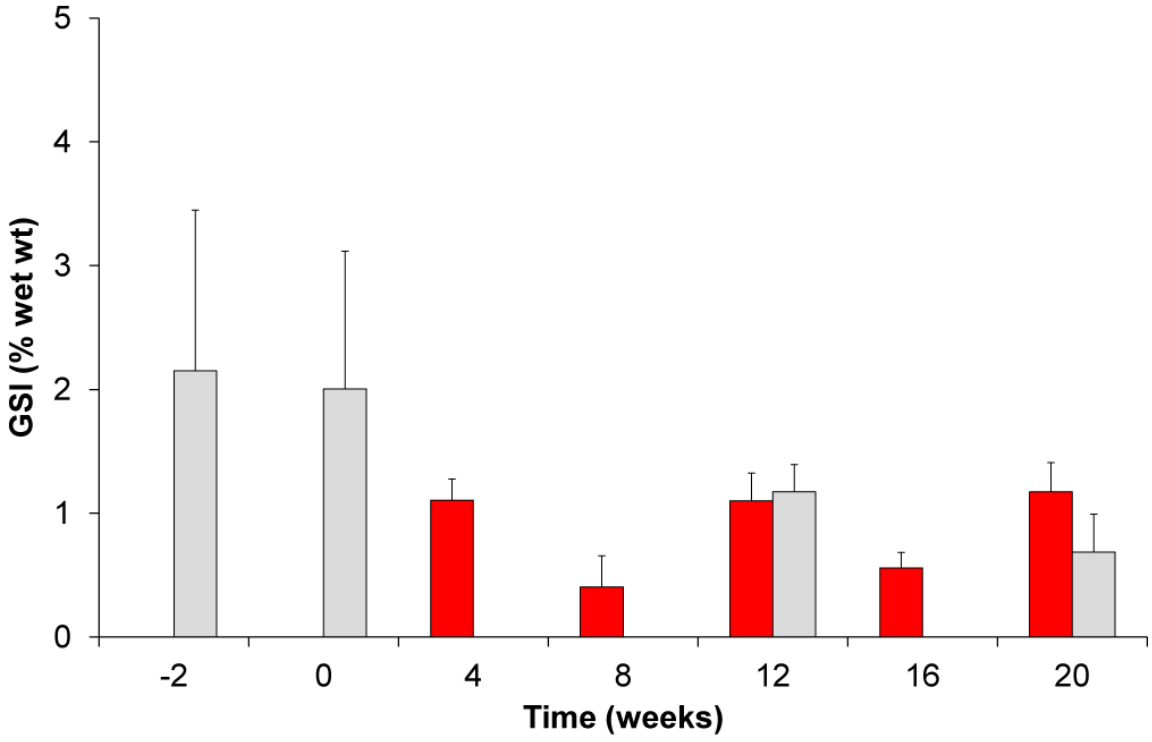

Figure 3.2 Gonadosomatic index (GSI) (means ± SE) in unfed (red bars) and fed (grey bars) groups of female *N. norvegicus* during the starvation trial. n=47

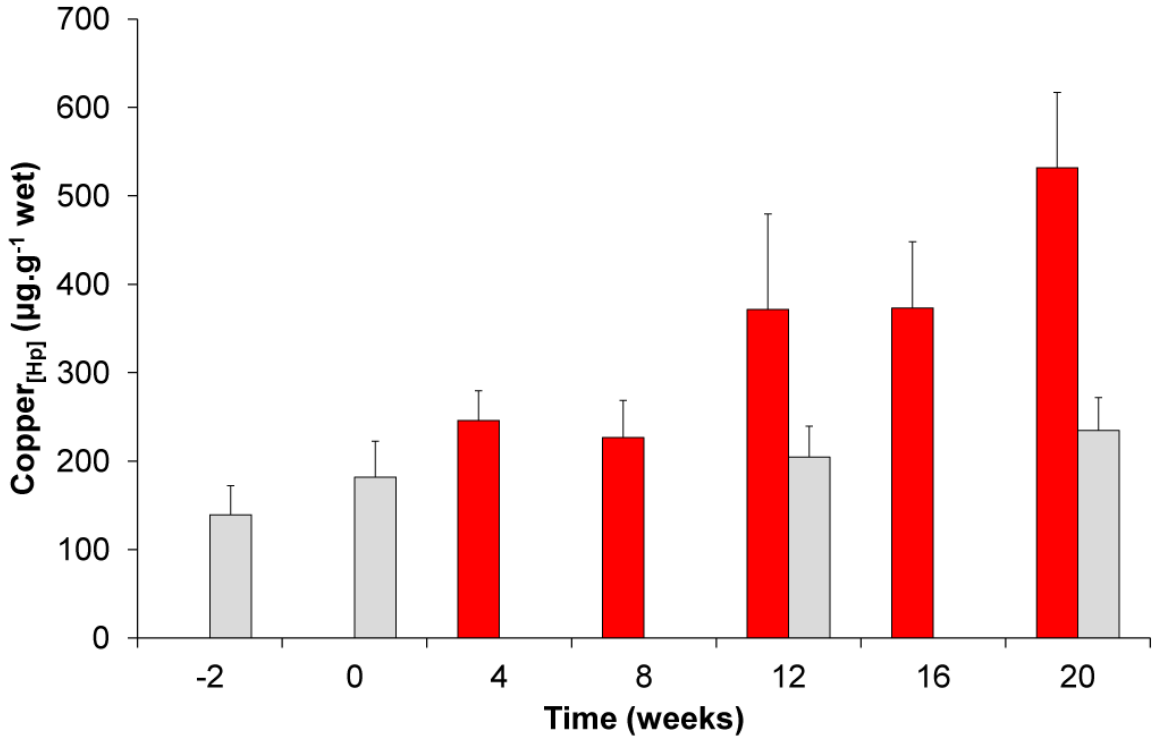

Figure 3.3 Copper concentration of the hepatopancreas (means ± SE expressed as wet weights) in unfed (red bars) and fed (grey bars) groups of female *N. norvegicus* during the starvation trial. n=47

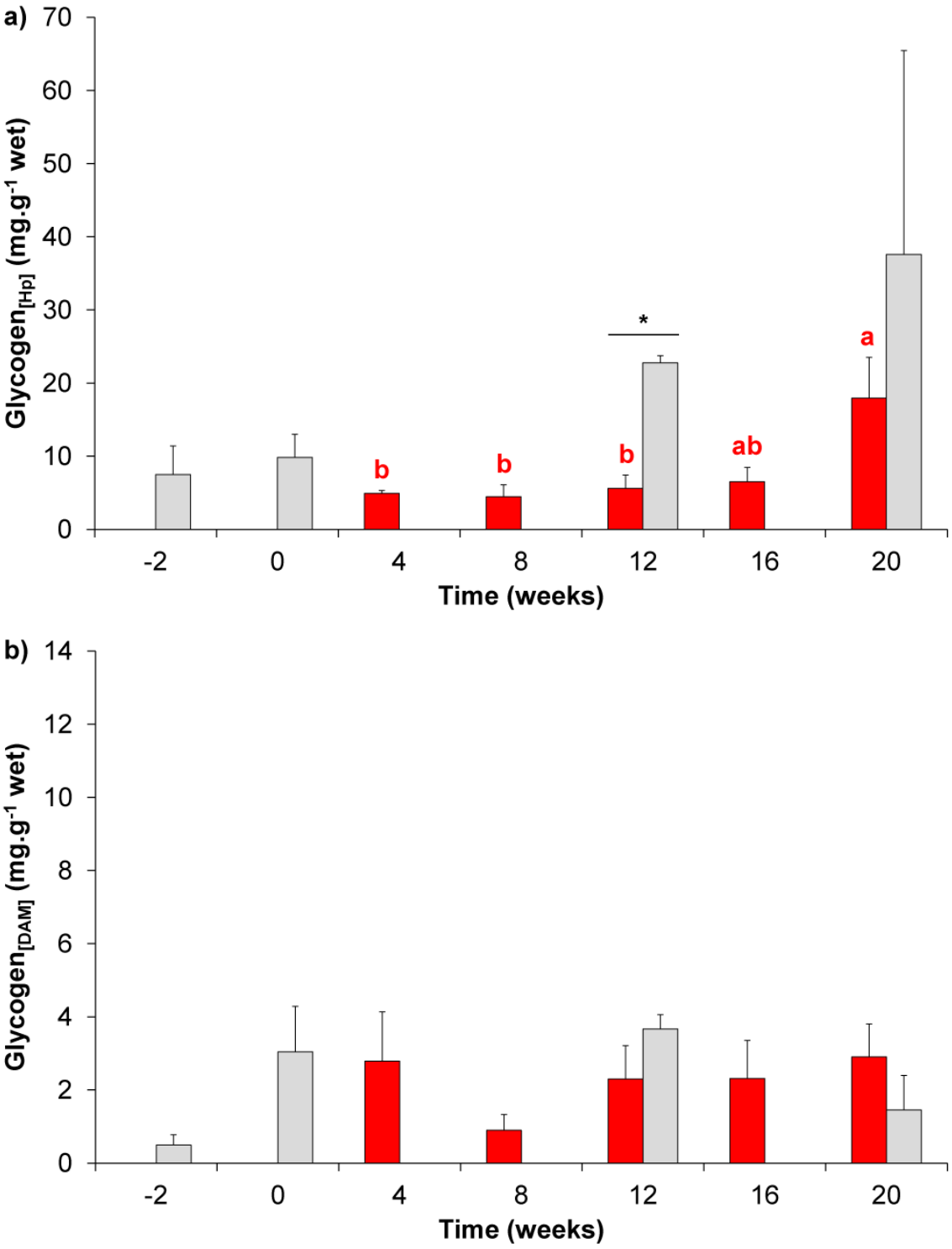

Figure 3.4 Glycogen concentration of a) hepatopancreas and b) tail muscle (means ± SE expressed as wet weights) in unfed (red bars) and fed (grey bars) groups of female *N. norvegicus* during the starvation trial. Asterisk represents a significant difference between fed and unfed groups ($P<0.05$). Red letters indicate the results of a post hoc Tukey test on unfed groups. Means that do not share a letter are significantly different ($P<0.05$). a) n=30, b) n=45

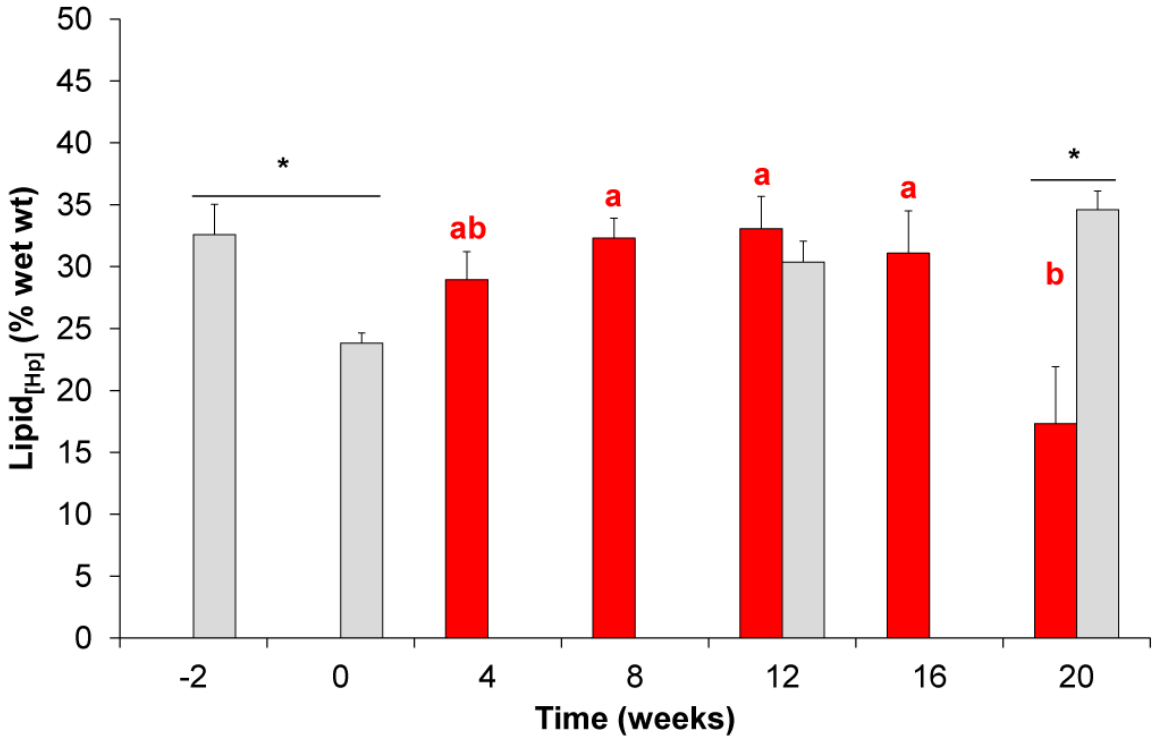

Figure 3.5 Lipid concentration of the hepatopancreas (means ± SE expressed as wet weights) in unfed (red bars) and fed (grey bars) groups of female *N. norvegicus* during the starvation trial. Asterisk represents a significant difference; between week -2 and 0 and between fed and unfed groups ($P<0.05$). Red letters indicate the results of a post hoc Tukey test on unfed groups. Means that do not share a letter are significantly different ($P<0.05$). n=37

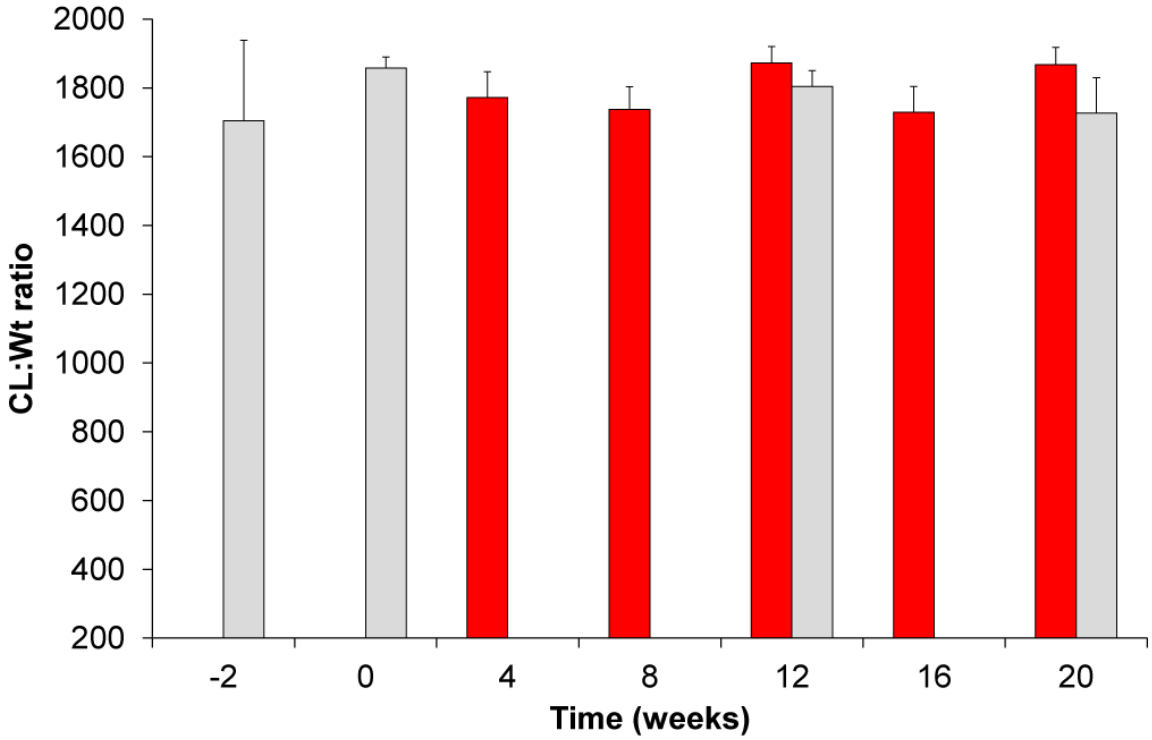

Figure 3.6 Carapace length:weight ratio (means ± SE) in unfed (red bars) and fed (grey bars) groups of female *N. norvegicus* during the starvation trial. n=47

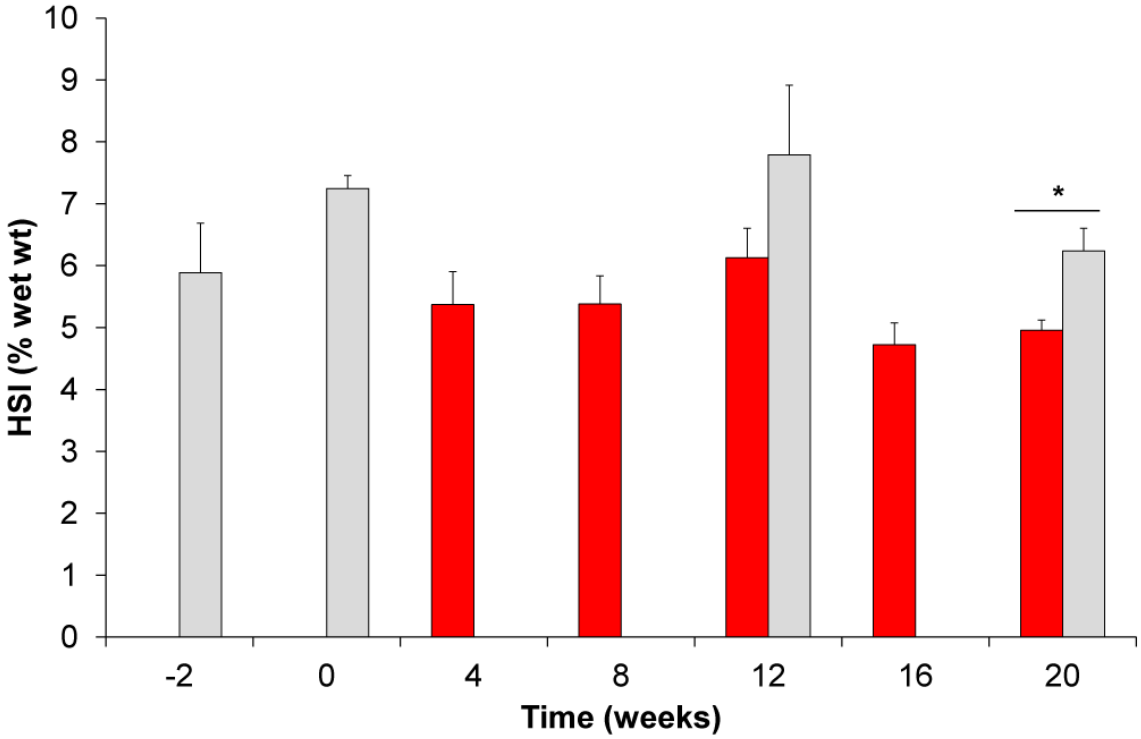

Figure 3.7 Hepatopsomatic index (HSI) (means ± SE) in unfed (red bars) and fed (grey bars) groups of female *N. norvegicus* during the starvation trial. Asterisk represents a significant difference between fed and unfed groups ($P<0.05$). n=47

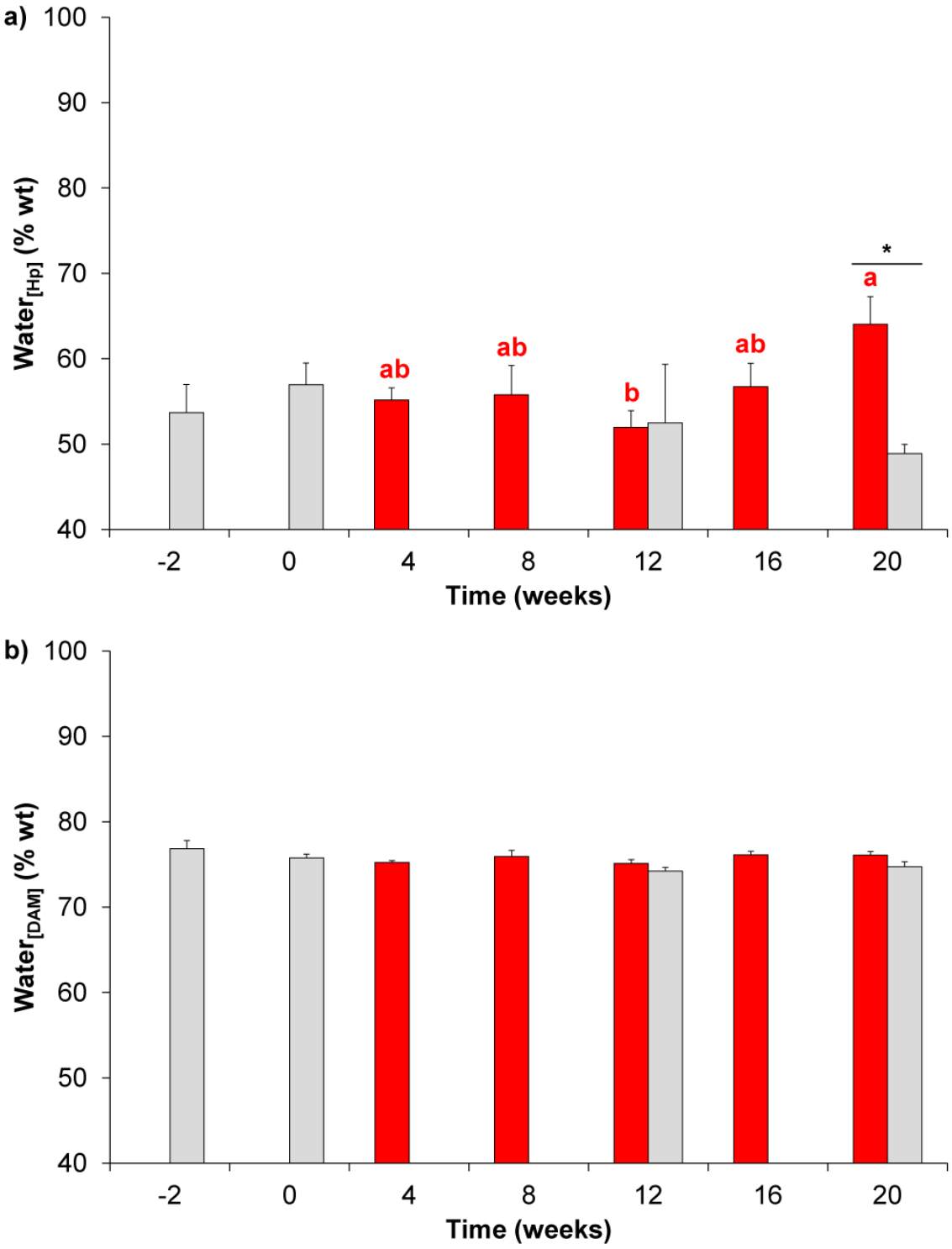

Figure 3.8 Water concentration of a) hepatopancreas and b) tail muscle (means ± SE) in unfed (red bars) and fed (grey bars) groups of female *N. norvegicus* during the starvation trial. Asterisk represents a significant difference between fed and unfed groups ($P<0.05$). Red letters indicate the results of a post hoc Tukey test on unfed groups. Means that do not share a letter are significantly different ($P<0.05$). n=47

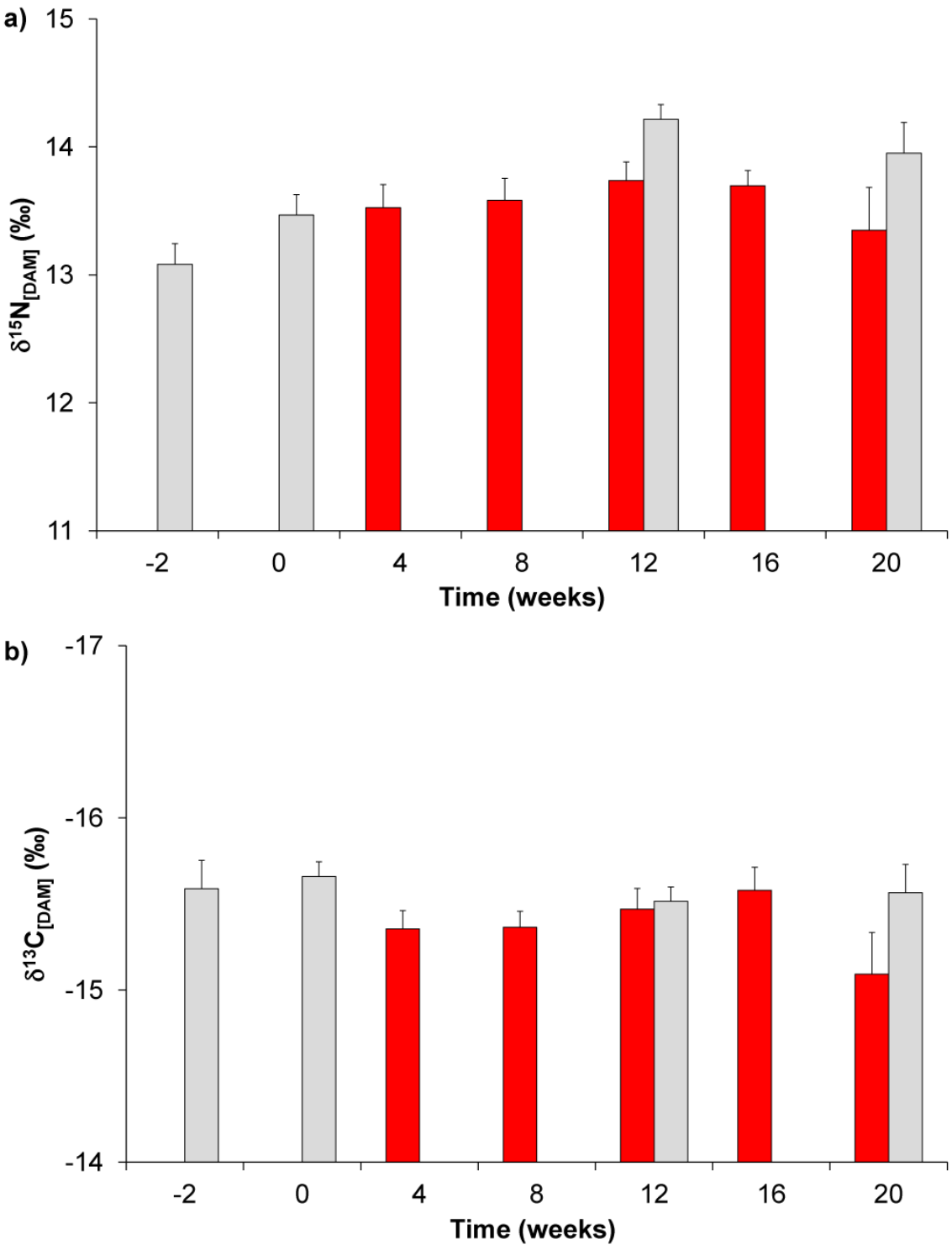

Figure 3.9 Stable Isotope of a) δ^{15}N and b) δ^{13}C in tail muscle (means ± SE) in unfed (red bars) and fed (grey bars) groups of female *N. norvegicus* during the starvation trial. n=47

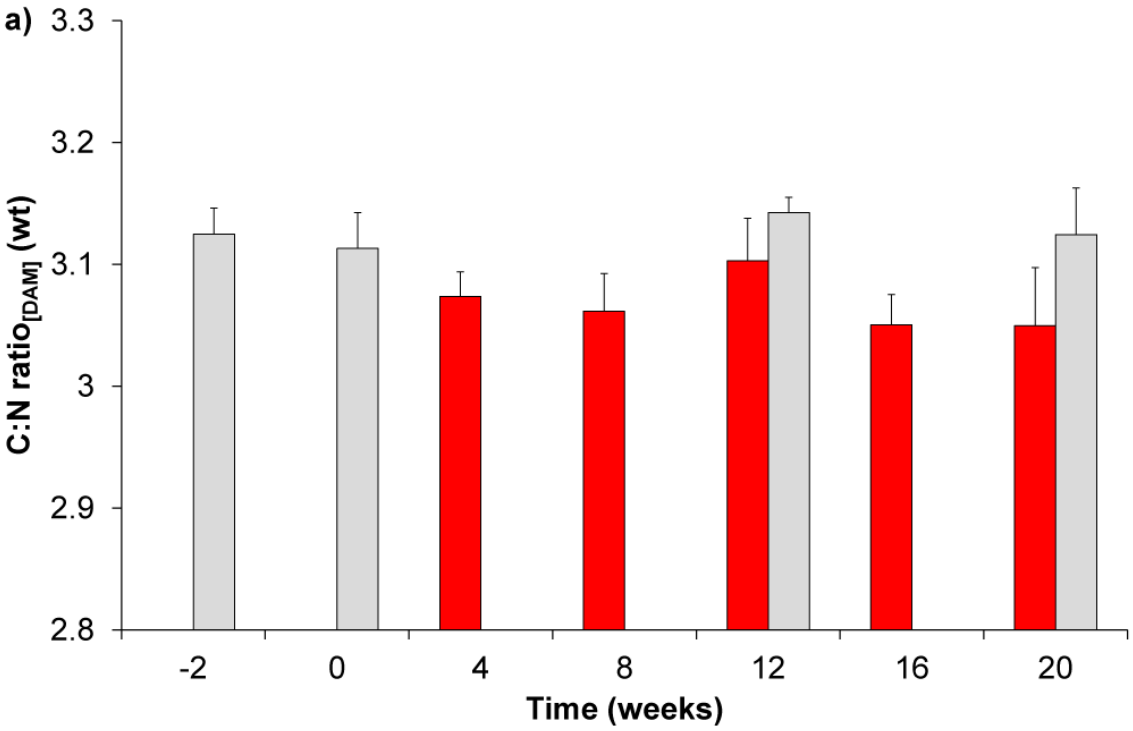

Figure 3.10 Carbon:Nitrogen ratio (C:N) of the tail muscle (means ± SE) in unfed (red bars) and fed (grey bars) groups of female *N. norvegicus* during the starvation trial. n=47

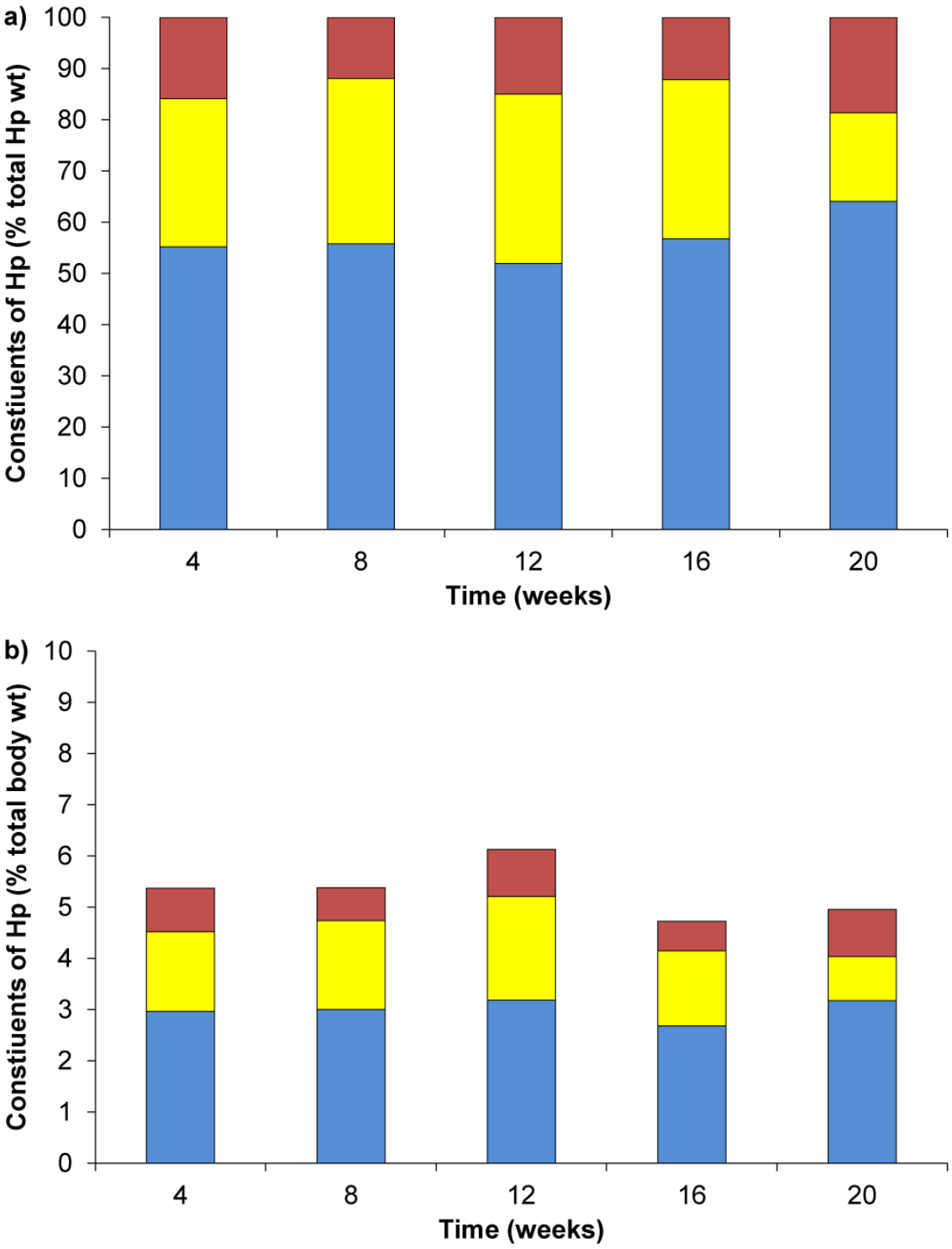

Figure 3.11 Time course of change in constituents of the hepatopancreas during the starvation trial: a) proportional to total hepatopancreas weight, b) proportional to whole body weight. Colour code: mid-blue is hepatopancreas water; yellow is hepatopancreas lipids; maroon is everything else (including protein, glycogen and copper). n=37

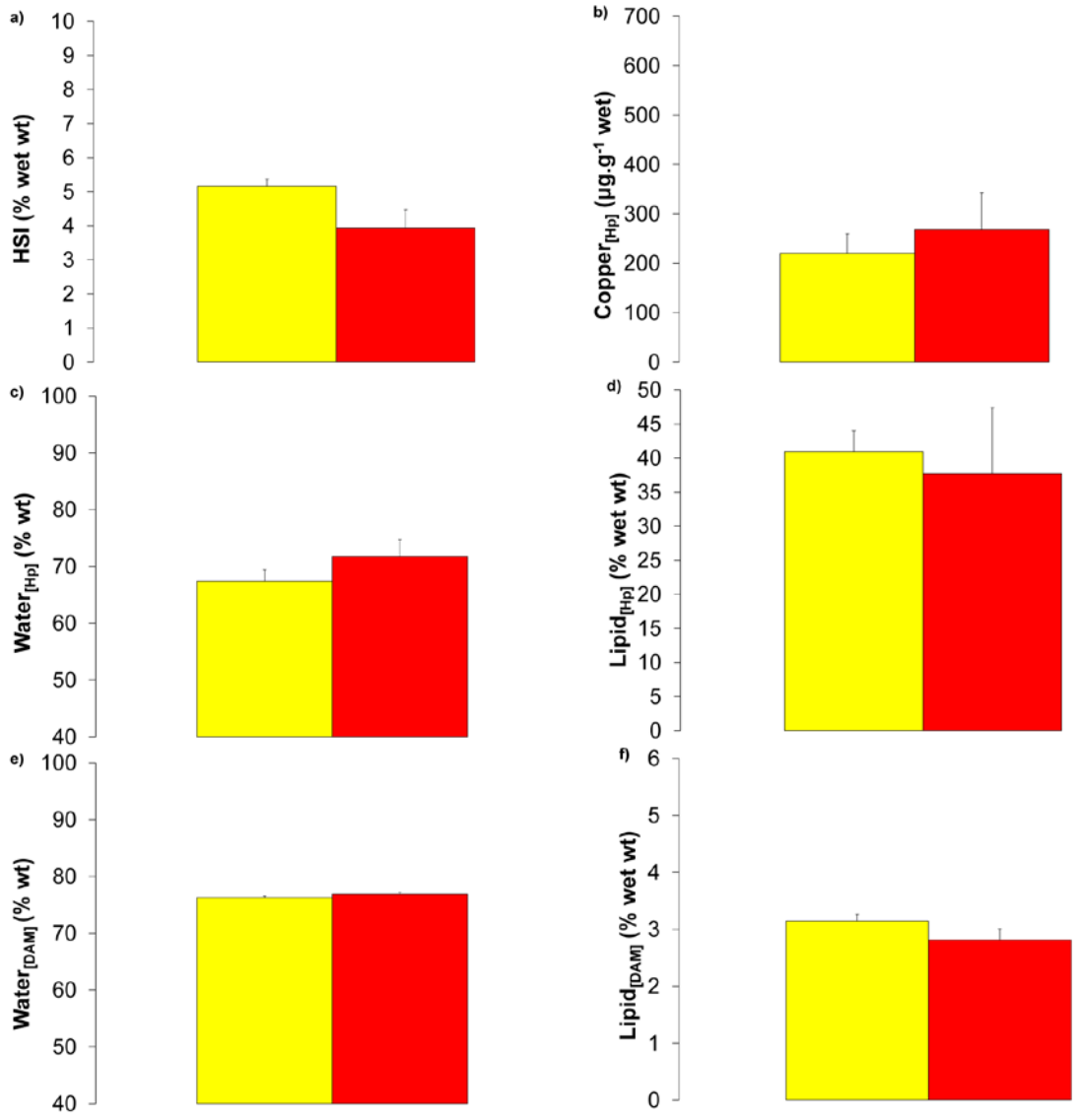

Figure 3.12 Parameters of previously unfed female *N. norvegicus* (means ± SE) after two different subsequent treatments: subsequently fed (yellow bars) and remaining unfed (red bars). Parameters as follows a) hepatosomatic index n=12, b) hepatopancreas copper n=10, c) hepatopancreas water n=12, d) hepatopancreas lipids n=9, e) tail water n=12, and f) tail lipids n=12.

4 Classification of starvation using critical threshold models to create usable biomarkers for field samples

4.1 Introduction

4.1.1 What are biomarkers?

A biomarker can be defined as a substance or measure that can be used as an indicator of a physiological condition (OED, 2012). The use of biomarkers involves more than simply measuring certain biochemical parameters within an organism. Since they can also be used quantitatively to define a biological state, in terms of either the actual biomarker concentration, or of an evaluation of whether this surpasses a defined threshold value.

Biomarkers have been used in many different areas of research, especially in the medical field. The earliest use of biomarkers in medicine was in medieval times, when urine colour, smell and taste were used to diagnose health (Koulman *et al.*, 2009). This has now developed into a sophisticated set of processes that use advanced bioinformatics to categorise medical conditions using such methods as proteomics (Kohn *et al.*, 2007), genomics (Sigdet & Sarwal, 2008) and metabolomics (Koulman *et al.,* 2009).

The other main use of biomarkers is in the field of environmental toxicology, for example for detecting an organism's response to pollution. Galloway (2006) describes two case studies involving different types of pollution biomarker. These are: discovering persistent organic pollutants such as polyaromatic hydrocarbons (PAHs) through measuring lymphocyte cytotoxicity in humans, mammals and fish; and detecting organophorous (OP) pesticides by studying the abundance of butyrylesterase activity in *Mytilus edulis*.

One frequently used biomarker, measuring aspects of nutrition, is that of the profile of the fatty acids in the tissues, which can be used to determine the types of food consumed. This will be discussed more extensively in Chapter 7.

Other biomarkers of nutritional status have been developed for human diagnoses, concentrating on non-invasive techniques such as measuring weight loss, dietary intake and urine measurements of iron, protein and other substances to identity different nutritional states (Blanck *et al*., 2003). In the present study the biological state under consideration is the nutritional status in *Nephrops norvegicus* and biomarkers are required to assess if the animal is in a starved state.

The starved state of an animal is defined as the time between absorption of its last meal and its subsequent meal. Initially the state will be undetectable and only when the biomarker used can distinguish this state from a normal control condition can the animal be confirmed as being in that starved state.

The concept of detection limits applies in all fields of experimental science and measures can only be expressed in relation to agreed standards for these. As an example, Small *et al.* (2002) used an ELISA (Enzyme-linked immunosorbent assay) method to detect the parasite *Hematodinium* infecting *N. norvegicus*. The detection limit of this method (5×10^4 parasites ml^{-1}) was determined experimentally by using serial dilutions of an original suspension of the parasites, until the results of the ELISA reaction failed to be statistically significantly different from a blank control. However, concentrations of the parasite within the animal below this limit still represent infection, despite being undetectable. As reported by Stentiford & Neil (2011), using molecular methods (PCR) with greater sensitivity than that of ELISA, the detection limit can be further reduced. However, intensities of infection expressed in relation to the less sensitive, but more practically useful ELISA methods still provide appropriate data for studying the epidemiology of this parasitic disease in different host populations (Beevers *et al.*, 2012).

Likewise in the present study, a starvation state could occur without being detected by current methods; however, a standard detection limit needs to be established to allow comparative measures on wild populations of *N. norvegicus* to be made.

4.1.2 Identifying appropriate biomarkers of starvation in *Nephrops norvegicus*

4.1.2.1 Traditional approach

Many studies have assessed certain measurements in *N. norvegicus* to detect starvation. These studies have either simply stated that certain parameters changed with starvation (Parslow-Williams *et al.*, 2001; Dall, 1981; Baden *et al.*, 1994; Jones & Obst, 2000; Mente, 2010) or that that certain reserves varied in amount through the year. IMBC *et al.* (1994) studied the levels of protein, carbohydrates, lipid and water content of both the abdominal muscle and the hepatopancreas of *N. norvegicus* over a year, based on quarterly sampling. Their results did not provide usable values to classify starvation, but instead only indicated trends in the parameters. Thus, for example, they found that lipids decreased with starvation, but they did not determine the level to which lipids should fall before the animal could be classified as being in a starved state.

4.1.2.2 New approach

Koulman *et al.* (2009) have suggested a set of standard procedures for biomarker discovery and validation. The focus of their work was on metabolomic biomarkers, but their procedures are valid more generally and have been adopted here. A schematic of this procedure can be seen in Figure 4.1. The first requirement is to identify the parameters that are altered significantly by a biological process such as disease, pollution or, as in the present case, starvation. This requirement has already been accomplished in the present study, as described in Chapters 2 and 3. Subsequent requirements include the first 'Qualification Stage', identifying the model biomarkers (i.e. how they will be measured and the type of model to be used), validation of the analysis (testing the model with the original data) and proofing the biomarker through a further study (i.e. testing the model with a separate set of data).

Biomarkers must also have the properties of 'precision' and 'robustness'. This means that on the one hand they need to be processes that truly reflect the state under consideration and on the other hand that they must be able to do

this independently of other changing factors and consistently in different individuals. A compromise between these different requirements needs to be struck and limitations of such a compromise need to be understood.

4.1.3 Aims

The aim of the present study was to apply these standard procedures to the validation of appropriate biomarkers for starvation in *N. norvegicus*. In the previous chapters data have been presented which show the effect of starvation on metabolic depression and the utilisation of reserves of *N. norvegicus*. The outcome of these studies was the identification of parameters that could potentially be used to determine the nutritional status of *N. norvegicus* obtained from the field. Each parameter could predict if an animal of unknown history was showing detectable signs of starvation. The parameters were selected based on three criteria:

1. There is a clear difference between fed and unfed animals.
2. This difference remains beyond a certain time point.
3. It is insensitive to short term stresses caused by the method of collection (e.g. trawl capture).

In this chapter two types of model will be tested. The first model will be a temporal prediction model, which yields a predicted duration of starvation. The second model will be the threshold model. This threshold was derived by determining the first week (critical time point - CTP) that unfed animals could be detected as being in a starved state, through being significantly different in a biomarker measure compared with all preceding weeks. All animals with values less than this (i.e. 0 to CTP-1) were grouped together and animals with values greater or equal to this (CTP+) were also grouped together. The CTP can therefore be considered to be the week in which animals can be detected to be in a starved state (or the detection limit).

To complete the validation, the candidate biomarkers will be tested to determine how well the model represents the original data. Finally, proof of the effectiveness of the model will be established through applying it to a second experimental data set (animals held under different conditions). Following this,

the processes for determining precision, robustness and limitations will be assessed, in the Discussion section.

4.2 Methodology

Data from the starvation trials performed with both male and female *N. norvegicus* (based on the data from Chapters 2 and 3) show that a significant difference develops in a number of parameters: namely HSI, hepatopancreas water, lipid and copper and the C:N ratio in the tail muscle for males; and HSI, hepatopancreas water, lipid and copper for females.

4.2.1 Discriminant analysis

Discriminant analysis is a multivariate tool which tests and predicts groupings of data in a model. It is used to estimate 'discriminant functions', which are linear combinations of the multiple variables that can be used to separate the data into different categories. Discriminant functions are estimated from a "training" data set. Further analysis can be performed on a "validation" dataset to test the reliability of these functions by using them to classify new items with known group membership. As the terms training and validation traditionally used in discriminate analysis can be confused when using the schematic of Koulman *et al.* (2009) (Figure 4.1), therefore for clarity the training data set will be expressed as Qualification Phase 1.2 'Validation of the analysis' and the validation data set will be expressed as Quantification Phase 2.3 'Proof of biomarker in new study'. In the present study the groupings are represented by time in weeks (for the temporal prediction model) or the critical time point (for the threshold model).

Each group is assigned coefficients for each factor discriminating between groups. These are known as the posterior probability. Each unidentified individual is assigned to the group with the highest posterior probability.

The terms used for the original model (applied to data obtained from a group of male animals- from the first and second male starvation trial) were HSI, hepatopancreas water, lipids and copper and the C:N ratio of the tail muscle.

Two types of discriminant analysis models, the temporal and the threshold models, will be evaluated, using the software Minitab.

4.2.2 Starvation threshold- (CTP)

For each parameter a 'starvation threshold' was estimated to indicate the expression of a detectable physiological effect of starvation from the CTP mentioned above.

Although different values of CTP may be obtained for the different parameters, all possible CTP values were compared for each biochemical measure and a GLM applied (Measure=separate group) to confirm that these groupings were statistically different from each other. The CTP derived from the groupings which significantly explained most variation in the data (largest r^2) was used for that measure.

Quartiles 1 and 3 of the distribution of each measure were determined for the CTP+ group and the values used to define the starvation threshold. Quartile 1 was used in cases where the measure of a parameter increased with starvation, e.g. hepatopancreas copper and water, and Quartile 3 was used where the measure of a parameter decreased with starvation, e.g. hepatopancreas lipids, HSI and the tail muscle C:N ratio.

4.3 Results

4.3.1 Discriminant analysis

4.3.1.1 Males

The coefficients of the discriminant analysis for both the temoral and threshold models can be seen in Table 4.1

The discriminant analysis for the temporal prediction model had an overall accuracy of 80% (Table 4.2a). All animals starved for 0, 12, 16 or 20 weeks were categorised in the correct group. Animals assessed after week 4 were predicted

to be in the correct time group in 80% of cases; while those assessed after week 8 were predicted to be in the correct time group in only 40% of cases.

In the threshold model, the discriminant analysis predicted that week 12 was the point at which male individuals could be detected as being in a starved state with an overall accuracy of 80%. Within the individual categories, the animals which were not fed for up to 8 weeks were correctly identified into the 0-8 group in 76.9% of cases and in the 12+ group were correctly identified in 85.7% of cases (Table 4.2b).

Table 4.1 The coefficients of the discriminant analysis, a) Temporal prediction model b) threshold model.

		Constant	HSI	Water	Copper	C:N	Lipids	r^2
a)	**0**	-2061.1	59.2	53.7	-0.2	-145.3	42.1	100%
	4	-1815.1	50.1	49.9	-0.2	-118.4	39.7	80%
	8	-1862.4	50.5	50.4	-0.2	-107.7	39.3	40%
	12	-1813.2	47.5	49.5	-0.2	-107.7	39.3	100%
	16	-1890.0	51.5	51.1	-0.2	-124.4	40.4	100%
	20	-2014.8	52.4	52.8	-0.2	-126.5	41.5	100%
b)	**0-8**	-1234.1	-5.2	28.3	0.0	65.2	28.2	77%
	12+	-1265.0	-6.1	28.8	0.0	62.4	28.7	86%

Table 4.2 The accuracy of each discriminant analysis to predict groups contained in the model (percentage correct). a) Temporal prediction model, b) Threshold (CTP) model

a) Temporal prediction model

week	Accuracy (%)
0	100
4	80
8	40
12	100
16	100
20	100
over all	80

b) Critical switch time point model

CTP	group	Accuracy (%)
	0-4	75
	8+	81.3
8+	over all	79.2
	0-8	76.9
	12+	85.7
12+	over all	80
	0-12	80
	16+	80
16+	over all	80
	0-16	88.9
	20+	100
20+	over all	90

4.3.1.2 Proof of biomarker in new study- Discriminant analysis

Both models derived from the discriminant analysis were then applied to the data set from the second trial on males, the accuracy of applying the coefficients (seen in Table 4.1) can be seen in Table 4.3. The CTP model allocated individuals to the 'not starved' (0–8 weeks) or 'starved' (12+ weeks) groups with an accuracy of only 52.94%. The temporal prediction model categorised animals in only two (week 0 or week 8) of the available six possible groups, and thus had an even lower accuracy of 29.41%.

Table 4.3 Testing the discriminant analysis with males. Bold: correct categorisations; red: incorrect categorisations

WEEK	fed	group prediction	week prediction
0	Y	**0-8**	**0**
0	Y	12+	**0**
0	Y	**0-8**	**0**
4	N	12+	0
4	N	**0-8**	8
4	N	12+	8
8	N	**0-8**	**8**
8	N	12+	0
8	N	**0-8**	0
12	N	**12+**	8
12	N	**12+**	0
12	Y	12+	**0**
12	Y	**0-8**	8
20	N	**12+**	8
20	N	0-8	8
20	Y	12+	8
20	Y	0-8	**0**
	r^2	**52.94**	**29.41**

4.3.2 Starvation threshold

4.3.2.1 Male starvation thresholds

Creating male starvation thresholds

The starvation threshold calculations from the first trial on males (summarised in Table 4.4) allowed the Critical Time Point (CTP) for males and then, in turn, the starvation threshold to be determined. Most parameters tested in males yielded a CTP of 12 weeks, apart from the C:N ratio which gave a CTP of 8 weeks.

The CTP for the copper concentration of the hepatopancreas in males which best classified starvation was week 12 (r^2=38.30%). The difference in the copper concentration of the hepatopancreas between week group 12+ and week group 0-8 was significant ($F_{1,23}$=13.7, P=0.001) and, thus, the proposed starvation threshold of the group 12+ according to the copper concentration in the hepatopancreas of males is 350.19 µg.g^{-1}.

The CTP for the lipid content of the hepatopancreas in males which best classified starvation was week 12 (r^2=21.99%). The difference in the lipid content of the hepatopancreas between week group 12+ and week group 0-8 was significant ($F_{1,23}$=18.02, P<0.001) and, thus, the proposed starvation threshold of the group 12+ according to the lipid content in the hepatopancreas of males is 8.30%.

The CTP for the water content of the hepatopancreas in males which best classified starvation was week 12 (r^2=53.41%). The difference in the water content of the hepatopancreas between week group 12+ and week group 0-8 was significant ($F_{1,28}$=30.95, P<0.001) and, thus, the proposed starvation threshold of the group 12+ according to the water content in the hepatopancreas of males is 68.64%.

The CTP for the HSI value in males which best classified starvation was week 12 (r^2=51.72%). The difference in the HSI between week group 12+ and week group 0-8 was significant ($F_{1,28}$=28.93, P<0.001) and, thus, the proposed starvation threshold of the group 12+ according to male HSI is 3.44%.

The CTP for the C:N ratio of the abdominal tail muscle in males which best classified starvation was week 8 (r^2=56.62%). The difference in the C:N ratio of the abdominal tail muscle between week group 8+ and week group 0-4 was significant ($F_{1,27}$=33.93, P<0.001) and, thus, the proposed starvation threshold of the group 8+ according to the C:N ratio of the abdominal tail muscle of males is 3.07.

Table 4.4 Starvation threshold statistics for male GLM between group weeks (0-[CTP-1] and (CTP+). n= total df + 1

	CTP	F	df	P	r^2 (%)	Median	direction	Starvation threshold
Copper ($\mu g.g^{-1}$)	8	6.67	1,23	**0.017**	23.3			
	12	13.7	1,23	**0.001**	**38.3**	444.49	Up	350.19
	16	1.94	1,23	0.177	8.12			
	20	0.05	1,23	0.824	0.23			
Lipids (% wt)	8	15.5	1,23	**0.001**	41.34			
	12	18.02	1,23	**<0.001**	**45.03**	3.22	Down	8.30
	16	9.18	1,23	**0.006**	29.45			
	20	6.48	1,23	**0.018**	22.75			
Water (% wt)	8	18.91	1,28	**<0.001**	41.19			
	12	30.95	1,28	**<0.001**	**53.41**	78.6	Up	68.64
	16	8.42	1,28	**0.007**	23.77			
	20	6.12	1,28	**0.02**	18.49			
HSI (% wt)	8	26.15	1,28	**<0.001**	49.2			
	12	28.93	1,28	**<0.001**	**51.72**	2.42	Down	3.44
	16	6.75	1,28	**0.015**	20.01			
	20	4.9	1,28	**0.036**	15.36			
C:N	**8**	33.93	1,27	**<0.001**	**56.62**	3.01	Down	3.07
	12	22.62	1,27	**<0.001**	46.53			
	16	11.99	1,27	**0.002**	31.57			
	20	4.24	1,27	**0.05**	14.01			

Testing male starvation thresholds

These starvation thresholds were then applied to the data from both trials on males. The results of the test can be seen in Table 4.5a, b. In the first trial the starvation thresholds placed individual animals in the correct group in 75.86% of cases for HSI, 79.31% of cases for hepatopancreas water content, 79.17% of cases for hepatopancreas lipid, 79.31% of cases for the tail muscle C:N ratio and 70.83% of cases for hepatopancreas copper.

When applied to the second set of data from males held under different conditions, the starvation thresholds of HSI, hepatopancreas copper and water predicted the correct group more accurately (above 80% accuracy).

When using the copper concentration of the hepatopancreas as a biomarker of starvation the animals were placed correctly into the CTP groups in 82.61% of the cases when using 0-8 and 12+ groups combined. Animals that had not been

fed for at least the CTP (12 weeks) were placed in this group according to the copper concentration of the hepatopancreas in 62.5% of the cases, while animals that had been fed over the period before the CTP (0-8 weeks) were placed in this group in 93.33% of the cases.

When using the lipid content of the hepatopancreas as a biomarker of starvation, the animals were placed outwith the CTP groups in 66.67% of the cases when using for 0-8 and 12+ groups combined. Animals that had not been fed for at least the CTP (12 weeks) were placed in this group according to the lipid content of the hepatopancreas in only 14.29% of the cases, while animals that had been fed over the period before the CTP (0-8 weeks) were placed in this group in 92.86% of the cases.

When using the water content of the hepatopancreas as a biomarker of starvation the animals was placed correctly outwith the CTP groups in 78.26% of the cases when using the 0-8 and 12+ groups combined. Animals that had not been fed for at least the CTP (12 weeks) were placed in this group according to the water content of the hepatopancreas in 62.5% of the cases, while animals that had been fed over the period before the CTP (0-8 weeks) were placed in this group in 86.67% of the cases.

When using HSI as a biomarker of starvation the animals were as placed in the correct groups outwith the CTP groups in 86.96% of the cases when using for 0-8 and 12+ groups combined. Animals that had not been feed for at least the CTP (12 weeks) were placed in this group according to the HSI in 62.5% of the cases, while animals that had been fed over the period before the CTP (0-8 weeks) were placed in this group in 100% of the cases.

When using the C:N ratio of the abdominal muscle as a biomarker of starvation, the animals were placed correctly outwith the CTP groups in only 52.94% of the cases when using for 0-4 and 8+ groups combined. Animals that had not been fed for at least the CTP (8 weeks) were placed in this group according to the C:N ratio in 85.71% of the cases, while animals that had been fed over the period before the CTP (0-8 weeks) were placed in this group in only 30% of the cases.

Table 4.5 Tests of the starvation thresholds, showng prcentage accuracy in terms of (CTP+), (0-[CTP-1]) and all groups combined. For a) first male study (which the starvation thresholds were derived from- Training data set 'validation of analysis'. b) testing these thresholds on the second male study which was carried out at a different water temperature- 'proof of biomarker in new study'.

				CTP+			0 - (CTP-1)			both
		CTP	ST	correct	all	Accuracy (%)	correct	all	percentage	percentage
a)	Copper	**12**	**>322.23**	8	10	80.00	9	14	64.29	70.83
	Lipid	**12**	**<12.06**	8	11	72.73	11	13	84.62	79.17
	Water	**12**	**>68.64**	11	15	73.33	12	14	85.71	79.31
	HSI	**12**	**<3.87**	11	15	73.33	11	14	78.57	75.86
	C:N	**8**	**<3.11**	15	20	75.00	8	9	88.89	79.31
b)	Copper	**12**	**>322.23**	5	8	62.50	14	15	93.33	82.61
	Lipid	**12**	**<12.06**	1	7	14.29	13	14	92.86	66.67
	Water	**12**	**>68.64**	5	8	62.50	13	15	86.67	78.26
	HSI	**12**	**<3.87**	5	8	62.50	15	15	100.00	86.96
	C:N	**8**	**<3.11**	6	7	85.71	3	10	30.00	52.94

4.3.2.2 Female starvation threshold

Creating female starvation thresholds

Table 4.6 shows the results from starvation threshold calculations to determine the CTP for female *N. norvegicus* and subsequently the starvation threshold.

The CTP for the copper concentration in the hepatopancreas of females which best classifies starvation is week 12 (r^2=28.19%). The difference between the values for hepatopancreas copper between week group 12+ and week group 0-8 was significant ($F_{1,35}$=13.34, P=0.001) and thus, the proposed starvation threshold of the group 12+ for the copper concentration in the hepatopancreas of females is 283.85 $\mu g.g^{-1}$.

The CTP for the lipid content of the hepatopancreas in females which best classifies starvation is week 20 (r^2=36.16%). The difference between the values for hepatopancreas lipid between week group 20+ and week group 0-16 was significant ($F_{1,28}$=15.29, P=0.001) and thus, the proposed starvation threshold of the group 20+ for the lipid content of the hepatopancreas in females is 26.83%.

The CTP for the water content of the hepatopancreas in females which best classifies starvation is week 20 (r^2=21.99%). This difference in the water content of the hepatopancreas between week group 20+ and week group 0-16 was significant ($F_{1,35}$=9.59, P=0.004) and thus, the proposed starvation threshold of the group 20+ for the water content in the hepatopancreas of females is 58.33%.

The CTP for the HSI in females which best classifies starvation is week 16 (r^2=21.42%). This difference in the HSI between week group 16+ and week group 0-12 was significant ($F_{1,35}$=9.27, P=0.004) and thus, the proposed starvation threshold of the group 16+ for female HSI is 5.41%.

Testing female starvation thresholds

As there were no repeat trials for females, Table 4.7 contains only the starvation thresholds tested, together with the data from which they were derived. The parameter that yielded the highest number of females in their correct groups (78.72% of cases) was the hepatopancreas copper concentration. In contrast, the HSI starvation threshold of the females was only 40.43% accurate.

Table 4.6 Starvation threshold statistics for female GLM between group weeks (0-[CTP-1] and (CTP+). Where n= the critical time point- the point where the measure had significantly risen or fallen from the values derived from the individuals scarified and measured at week 0. n= total df + 1

	CTP	F	df	P	r^2 (%)	Median	direction	Starvation threshold
Copper (µg g^{-1})	8	6.13	1,35	**0.018**	15.28			
	12	13.34	1,35	**0.001**	**28.19**	379.34	Down	283.85
	16	9.80	1,35	**0.004**	22.38			
	20	10.23	1,35	**0.003**	23.13			
Lipids (%)	8	0.37	1,28	0.547	1.36			
	12	0.24	1,28	0.627	0.89			
	16	4.02	1,28	0.055	12.96			
	20	15.29	1,28	**0.001**	**36.16**	17.13	Down	26.83
Water (%)	8	0.18	1,35	0.674	0.53			
	12	0.47	1,35	0.499	1.35			
	16	5.36	1,35	**0.027**	13.61			
	20	9.59	1,35	**0.004**	**21.99**	62.44	Up	58.33
HSI (%)	8	6.21	1,35	**0.018**	15.44			
	12	3.38	1,35	0.075	9.04			
	16	9.27	1,35	**0.004**	**21.42**	4.96	Down	5.41
	20	2.26	1,35	0.142	6.23			

Table 4.7 Testing starvation threshold, using starvation threshold to predict which group the individuals belong to via each measure testing female starvation thresholds from the female study (from which the starvation thresholds were derived).

			CTP+			0- (CTP-1)			both
	CTP	**ST**	**correct**	**all**	**percentage**	**correct**	**all**	**percentage**	**percentage**
Copper	**12**	>283.85	23	29	79.31	14	18	77.78	78.72
Lipids	**20**	<26.83	21	33	63.64	4	5	80.00	65.79
Water	**20**	>58.33	21	41	51.22	5	6	83.33	55.32
HSI	**16**	<5.41	10	35	28.57	9	12	75.00	40.43

4.4 Discussion

Following the scheme of Koulman *et al.* (2009) for biomarker discovery and validation, as outlined in Figure 4.1, the process of identifying parameters of starvation described in Chapters 2 and 3 can be considered the discovery phase. The aim of the present chapter was then to carry out phases 1 and 2 of the subsequent 'qualification' phase.

4.4.1.1 Discriminant analysis

The first model, created using discriminant analysis, was a temporal prediction model and the second was a critical switch time point model. Of these two models the temporal prediction model was more accurate than the CTP model when using discriminant analysis, with an overall accuracy of 80% compared to 52.94% in the CTP.

The temporal prediction model, however, correctly classified animals sacrificed at week 8 in only 40% of cases; this was just before the parameters that were measured in animals were significantly different from animals measured at week 0. Consequently, when applied to the data from the second trial with males, the model classified the starvation period of the animals as only week 8 or week 0, irrespective of the time since food withdrawal. This means that this type of model fails as a predictive device.

Using the second (CTP) model, four different critical switch time points were tested to determine which had the greatest accuracy (8+, 12+, 16+ and 20+).

Both the time points 12+ and 16+ had an overall accuracy of 80%, with time points 12+ for the unfed group having the greatest accuracy (85.7%). As this model has only two possible outcomes (0-8 or 12+) it is less likely to be skewed by one outcome. The group prediction was correct 52.94% of the time.

The main weakness of discriminant analysis is that it gives a definite grouping to every individual with no confidence in this given and therefore it is possible that animals can be incorrectly categorised as being in a starved state or not.

As both these models had such low utility, no further use was made of them to test other groups, e.g. females.

4.4.1.2 Starvation threshold

The effectiveness of using the CTP model to determine the starvation threshold model was evaluated. To do this different critical time points were tested via a GLM, in which the parameter was the response and the groupings of 0-[CTP-1] and CTP+ were the categorical explanatory variables. In the male trial most CTP values were determined as 12+, being the best CTP due to having the largest r^2 values, which is in line with the findings of the previous chapters. The CTP for C:N ratio however was 8+, as this had a larger r^2 value than 12+.

In females the CTPs derived from the different hepatopancreas parameters did not converge on a single time point. Thus, copper gave 12+, HSI gave 16+ and water and lipids both gave 20+. This was due to the slower reaction to starvation seen in the female trial compared with the male trial as explained in Chapter 3. The groups in the female trial explained less variation in all the biomarkers than in males (lower r^2 values), meaning that less confidence can be placed on the female starvation thresholds than on those of the males.

The starvation thresholds derived allow those animals showing signs of starvation to be identified (i.e. last meal <CTP weeks) or not fed for at least CTP weeks.

Male starvation thresholds were also tested against data from the second male trial. Here HSI (86.96%) and hepatopancreas copper (82.61%) and water (78.26%) gave the highest accuracies in predicting the starved state. However, the C:N

ratio had an equivalent accuracy of only 34.78%, with all animals being categorised as not fed for 12 weeks or more. This is due to the C:N ratios being much lower in the second trial. Similarly the starvation thresholds of lipid correctly predicted the state of all the animals 66% of the time. However, when comparing the animals that should be placed in the 12+ group, there was only a 14.29% accuracy, indicating that lipid content in animals from the second male trial was higher than animals measured in the first male trial. This suggest that the C:N ratio and hepatopancreas lipid are affected by additional factors other than starvation and that they, therefore, do not fulfil the criterion of being specific to starvation. These other possible factors include temperature (as discussed in Chapter 3) and seasonal change in the affected parameters: hepatopancreas lipid and the C:N ratios.

In summary, it seems that the starvation threshold model has the greater utility. For males, the thresholds for HSI, hepatopancreas copper and water are the best predictors of starvation.

4.4.2 Qualification phase 2

Following the discovery phase and qualification phase 1, the next set of standard processes suggested by Koulman *et al.* (2009) for establishing a biomarker (qualification phase 2) is to assess its precision, robustness and limitations of its use.

To assess the precision of the biomarkers, further experiments would need to be conducted with different parameters, for example a wider range of temperatures, salinities, oxygen saturation levels, matching their ranges in the natural habitat of the animals.

To assess robustness, factors other than starvation that are known to affect the biochemistry of *N. norvegicus*, such as moulting condition and parasitic infection, need to be controlled for. These are considered further below.

4.4.2.1 Moulting

The period around the moulting event (ecdysis) is a period of substantial physiological change in *N. norvegicus.* Prior to the moult, organic reserves are accumulated during intermoult and premoult (Aiken, 1980). Water is likely to increase immediately postmoult as the animal expands in size before the exoskeleton is calcified. Lipid in the midgut increases in premoult crayfish and land crabs (Chang, 1995), with the lipids used in the moult cycle decreasing immediately postmoult (Alvarez-Fernandez *et al.*, 2005). Chang (1995) suggested that haemocyanin is low in postmoult animals, which could lead to an increase in the copper concentration of the hepatopancreas. During ecdysis there is no oxygen consumption by the animal (Alcaraz & Sarda, 1981). Moreover, Engel & Bouwer (1993) showed that copper in the digestive gland (hepatopancreas) and the haemolymph of the blue crab *Callinectes sapidus* decreases considerably immediately after ecdysis. This is followed by a spike at postmoult, although this is only to around half the value that occurs in animals in the intermoult state.

4.4.2.2 Parasitism

Infection of *N. norvegicus* by the dinoflagellate *Hematodinium* sp. has been shown to occur at various locations along the west coast of Scotland (Field *et al.*, 1992 & 1998; Field & Appleton, 1995; Stentiford *et al.*, 2001a,b; Stentiford & Shields, 2005; Hamilton *et al.,* 2010; Stentiford & Neil, 2011; Beevers *et al.*, 2012). Field *et al.* (1992) and Beevers *et al.* (2012) showed that the peak period of the prevalence of this parasite in the CSA was in the months February to May and that females have also been shown to have higher prevalence than males. Field studies of *N. norvegicus* at these locations would almost certainly encounter infected animals and any biochemical parameters measured to assess starvation could, therefore, be affected by this parasitism.

At the peak of its infection intensity (patency) the *Hematodinium* parasite places an enormous physiological demand on the host (Stentiford & Shields, 2005) including effects on the haemolymph, tail muscle and hepatopancreas. The parasite affects the structural integrity of these tissues, making extraction and weight determination difficult. The water content of these tissues is also

increased as the structure is degraded. All these factors will affect measures being made to assess the starvation state of the host.

Taylor *et al.* (1996) showed that *N. norvegicus* with patent *Hematodinium* infection had increased oxygen consumption, mainly due to the respiratory demands of the parasites. However, there was a 50% reduction in the oxygen carrying capacity of the host haemolymph, due to a lowered haemocyanin concentration in the haemolymph. This would be expected to lead to an accumulation of copper in the hepatopancreas as the haemocyanin was broken down. Such effects could obviously compromise the use of hepatopancreas copper as a biomarker for starvation.

Hematodinium infection of *N. norvegicus* is also known to disrupt the endocrine control of glycogen mobilisation from the hepatopancreas and tail muscle (Stentiford *et al.*, 2001a). As a result, these carbohydrate stores are completed depleted and are consumed mainly by the parasites. Such an effect would completely mask any changes in carbohydrates due to starvation of the host, *per se*.

These considerations about moulting and parasitism have been taken into account in the field studies to be reported in Chapter 5 on the nutritional status of *N. norvegicus* at the two study sites on the west coast of Scotland throughout the year. They were controlled for by checking that sampled animals were in the intermoult condition (if in large enough numbers) and that none was expressing patent *Hematodinium* infection (i.e. was not visually detectable). In this way it was more certain that starvation was the main factor being assessed through the measured parameters and the application of the starvation threshold (ST) model.

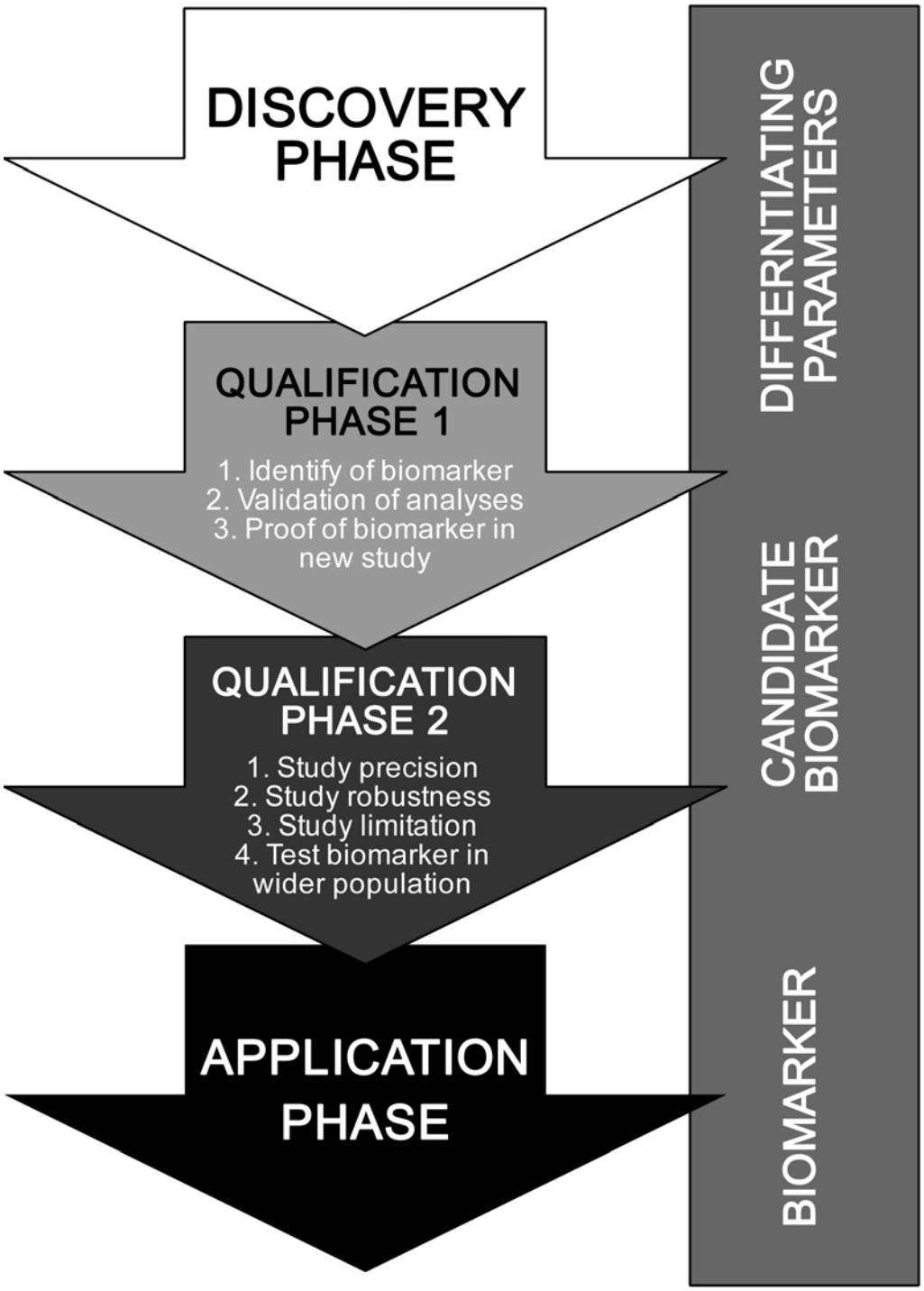

Figure 4.1 Schematic diagram showing the sequence of procedures for validating biomarkers. After Koulman *et al.* (2009).

5 Seasonal variation in the nutritional status of *Nephrops norvegicus* in the field

5.1 Introduction

5.1.1 Natural nutritional status variation

The variation in the nutritional status of animals in their natural environment is likely to be affected by a number of factors, such as the sex of the animal, the reproductive state of females, the point in the moulting cycle and seasonal changes in food availability. Site conditions dictate the amount of food available for capture through predation pressures, density-dependant factors or simply the amount of food in the reachable environment.

5.1.2 Nutritional status measurements

In Chapters 2 and 3 various parameters were measured in *Nephrops norvegicus* that had been starved for different periods of time, to see if they were markers of starvation. This showed that HSI, water, and lipid content and copper concentration of the hepatopancreas, along with the C:N ratio of the tail muscle, were all measurements that could be used to help determine the nutritional status of an individual *N. norvegicus*. Chapter 3 noted that there were different effects of these measurements in the two sexes, with females retaining more resources for longer than males (possibly due to an effect of the reproductive cycle or as an effect of temperature).

In Chapter 4 these measurements were then converted into a starvation threshold (ST) at which point, if certain assumptions are made, an animal was considered to be in a significantly starved state. HSI, lipid content of the hepatopancreas and the C:N ratio of the tail muscle all decreased as a response to starvation, whereas the water content and copper concentration of the hepatopancreas increased as a response to starvation. The ST for males and females for each measure are shown in Table 5.1.

Table 5.1 Starvation thresholds for the 5 measurements used in this study. Direction indicates if the measure has to be higher or lower than the ST to indicate a reduced nutritional status. For females no ST was derived for the C:N ratio in the tail muscle as there was no significant drop in this measure within the first 20 weeks of food withdrawal. HSI, lipid and copper concentration of the hepatopancreas are displayed in percentage of wet weight.

	Direction	Males	Females
Hepatopancreas copper ($\mu g.g^{-1}$ wet)	Higher	350.19	283.85
Hepatopancreas lipid (% wet wt)	Lower	8.30	26.83
Hepatopancreas water (% wt)	Higher	68.64	58.33
HSI	Lower	3.44	4.69
tail muscle C:N ratio	Lower	3.07	n.d.

5.1.3 Aims

5.1.3.1 Sex specific interactions

As explained in Section 1.4 of Chapter 1, the proportions of male and female *N. norvegicus* caught by trawling alter seasonally. The measurements of nutritional status will show whether or not the females which have recently emerged from winter period of burrow residence show any detectable signs of being in a starved state and if this status is significantly different from the nutritional status of males, which are presumed to be able to forage as normal throughout the winter.

5.1.3.2 Seasonal interactions

As explained in Section 1.5 of Chapter 1, food availability may alter seasonally, ultimately due to the reduction in energy sinking to the benthos mainly in the form of phytoplankton. The measurements of nutritional status will show if food availability is constant throughout the year or if there is a reduction.

5.1.3.3 Site interactions

As explained in Section 1.6 of Chapter 1, the densities of *N. norvegicus* in different areas affect their nutritional status, with higher burrow densities

leading to greater competition for food and thus a lower nutritional status (Parslow-Williams 1998). The Clyde Sea Area (CSA) in 2009 had a mean *N. norvegicus* burrow density of 0.85 m^{-2} compared with the North Minch (NM) with a burrow density of 0.55 m^{-2} (ICES, 2011). According to this difference in density, it seems the *N. norvegicus* in the CSA may have a lower nutritional status than those in the NM.

5.2 Materials and Methods

5.2.1 Sample collection and processing

Starting in January 2009, *N. norvegicus* were collected monthly from trawl catches in the Clyde Sea Area, Scotland, UK (CSA) along both transects shown in Figure 1.5b. Three research vessels were used throughout the sampling period (RV Aora, RV Aplysia, and RV Actinia*)* (UMBSM), each equipped with an otter trawl fitted with a net of mesh size 70 mm at the cod-end. Starting in February 2009, *N. norvegicus* were also collected bi-monthly from single trawl catches in the North Minch, Scotland, UK (NM) along 2 transects shown in Figure 1.5a. The fishing vessel MFV Comrade III, equipped with an otter trawl fitted with a net of mesh size 70 mm at the cod end, was used throughout the sampling period.

The trawls along the two transects in the CSA each lasted for 1 h, at a mean depth of 78.5 ± 1.1 m for the north transect and 86.7 ± 1.7 m for the west transect. The trawls along the two transects in the NM each lasted for 2 h, at a mean depth of 116.96 ± 0.48 m for the east transect and 120.92 ± 2.86 m for the south transect.

5.2.1.1 Sub-sampling the catch

A shovelled sub-sample of *N. norvegicus* (*ca* volume of one fishing basket) was collected from each catch before any other animals were removed for other purposes. The bycatch was removed and discarded, leaving approximately 200 *N. norvegicus* (depending on the size of the catch). These animals were covered with ice, transported back to the University of Glasgow and then kept frozen at -20˚C until used for further processing.

Carapace length, weight without chelipeds (to avoid differences due to claw loss during trawling and post-capture handling), and sex were recorded for all of these animals using procedures described in previous chapters. When the catch had fewer than 200 animals the entire catch was recorded. For a subset of 25 males and 25 females the colour and weight of the hepatopancreas, and the colour and weight of the ovaries in females, were recorded.

5.2.1.2 Animals for nutritional status measurements

Fifteen male and fifteen female *N. norvegicus* were then collected from the remainder of the catch from each transect. Their moult stage, according to carapace hardness, was determined as pre/post moult (soft), immediate post moult (jelly) or intermoult (hard). Hard animals were taken preferentially, and jelly animals were used only when low numbers of animals were caught. The carapace length, weight, hepatopancreas colour and weight, and gonad colour, stage and weight were recorded. Samples of tail muscle and hepatopancreas were then taken from the 30 individuals sampled, flash frozen in liquid nitrogen and transported back to the University of Glasgow where they were stored at -80°C until processed further.

5.2.1.3 Targeted catch to test effect of maturity on nutritional status

Separate samples were taken from the north transect of the CSA in June (1^{st}-8^{th}-9^{th}-24^{th}) and December (8^{th}) 2010 to represent the summer and winter periods of the year. Carapace length, body weight (less chelipeds), sex, hepatopancreas weight and gonad weight were recorded in all samples caught, allowing HSI and GSI to be determined for each animal.

5.2.2 Measurements

5.2.2.1 Biometric measurements

In the sub-sampled catches the sex ratio, body size (as CL), body weight (less chelipeds), HSI and GSI were determined. The HSI and GSI values were used for the determination of sexual maturity, as appropriate.

5.2.2.2 Measurements made to determine nutritional status

In the processed samples the HSI was measured, and the amounts of water, lipid and copper in the hepatopancreas, as well as the C:N ratio of the tail muscle, were determined. All protocols were the same, as described in Chapter 2, Section 2.2. HSI was not measured in the NM animals caught to measure nutritional status; therefore site compositions of HSI will be from the biometric measurements from the sub-sampled catches.

5.2.3 Statistical analysis

The data were analysed, where appropriate, with a general linear model, and a subsequent post hoc Tukey test on the interactions (when significant). Normality of residuals was assessed visually, and a square-root transformation was applied when normality was not met. The terms for all the statistical tests described in this chapter are shown in Table 5.2.

Total number of observations in each table and figure where appropriate are reported as n=# or (when statistical output is present in tables) n=total df + 1, a breakdown of numbers of observations in each factor is displayed within the appendices.

Table 5.2 Terms for the statistical tests used in this chapter

Variable	Type of variable	Number of categories	Description of variable
Site	categorical	2	Clyde Sea Area (CSA) North transect and North Minch (NM) East transect
Month	categorical	12	All months in 2009
Time of Year	categorical	2	2009 split in to Jan-June and July-Dec
Sex	categorical	2	Male and Female
Eggs (females only)	categorical	2	With and without
Gonad stage (females only)	categorical	8	Stages 1-8 (as described in Table 1.1)
Gonad group (females only)	categorical	3	Stages 1-8 grouped into 3 categories : A (8,1,2), B (3,4), C (5,6,7)
Maturity (females only)	categorical	2	Immature and mature
Carapace length	continuous	-	The length of carapace between the eye socket and posterior margin of carapace
Hepatopancreas copper ($\mu g.g^{-1}$ wet weight)	continuous	-	**Parameter-** The amount of copper in the hepatopancreas expressed as $\mu g.g^{-1}$ wet weight
Hepatopancreas lipid (% wet weight)	continuous	-	**Parameter-** The amount of lipid in the hepatopancreas expressed as percentage weight
Hepatopancreas water (% weight)	continuous	-	**Parameter-** The amount of water in the hepatopancreas expressed as percentage weight
HSI (% wet weight)	continuous	-	**Parameter-** The proportion of the whole animal weight that is hepatopancreas expressed as a percentage
Tail muscle C:N ratio	continuous	-	**Parameter-** The ratio of carbon and nitrogen in the tail muscle, expressed as a ratio by weight

5.2.4 Representative sites

In order to make the task of analysis of all the biochemical measurements indicative of nutritional status a feasible one it was necessary to restrict the number of animals sampled, while retaining a comparison between the two main sites (the CSA and the NM). Based on the completeness of the data sets for the two transects from each sample site (Table 5.3), the north transect was selected to be representative of the Clyde Sea Area (and hereafter in this chapter will be referred to as the CSA), and the east transect was selected to be representative of the North Minch (and hereafter in this chapter will be referred to as the NM). As the NM bi-monthly data started in February, and since only one female was obtained in the north transect of the CSA in January, it was decided not to

include the January CSA data in the analysis. The other two transects will be used to compare sex ratios and will be used in Chapter 6.

5.3 Results

A total of 7242 *Nephrops norvegicus* were collected as sub-sampled catches throughout the year of 2009. A further 1010 animals were processed for their nutritional status. For the maturity analysis, 352 animals were assessed in July 2009 and 98 animals were assessed in December 2009. A summary of the monthly numbers is shown in Table 5.3.

Table 5.3 The number of animals from the two study sites: a) CSA and b) NM, that were used in the analyses reported in this chapter.

	CSA- north transect				**CSA- west transect**			
	subsample		**processed**		**subsample**		**processed**	
a)	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
Jan-09	37	1	15	1	55	7	15	15
Feb-09	121	51	15	15	147	39	15	15
Mar-09	139	68	15	15	74	31	15	15
Apr-09	76	77	15	15	145	82	15	15
May-09	107	204	15	15	151	234	15	15
Jun-09	83	159	15	15	80	176	15	15
Jul-09	40	95	15	15	64	118	15	15
Aug-09	69	66	15	15	141	95	15	15
Sep-09	49	35	9	11	88	93	10	10
Oct-09	82	15	15	15	46	17	15	15
Nov-09	249	40	15	15	-	-	-	-
Dec-09	249	40	15	15	140	23	15	15
	NM- east transect				**NM- south transect**			
	subsample		**processed**		**subsample**		**processed**	
b)	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
Feb-09	179	76	15	15	225	83	15	9
Apr-09	252	47	15	15	178	120	15	15
Jun-09	84	231	15	15	116	324	15	15
Aug-09	23	35	15	15	72	50	15	15
Oct-09	125	31	15	15	160	56	15	15
Dec-09	278	61	15	15	205	33	15	15

5.3.1 The proportion of females in the trawl catches

The seasonal pattern in the proportion of females in the trawl catches from each transect in both areas are shown in Figure 5.1. The main features for females were low numbers during the winter months, an increasing proportion in the spring, peak abundance in the summer, a declining proportion in the autumn and a return to low numbers in the winter.

5.3.2 Sex and Month interactions

The copper, lipid and water content of the hepatopancreas, the HSI and the C:N ratio of the tail muscle were measured in the animals from the CSA and the NM separately. An overview of all statistical results is presented in Table 5.4. If the effect of the interaction of sex and month are significant then the means are displayed in Table 5.5 and if not significant and month is significant then the means are displayed in Table 5.6

5.3.2.1 Copper concentration in the hepatopancreas

The mean values (± SE) for the copper concentration in the hepatopancreas of male and female *N. norvegicus,* within each sample selected from the monthly catches of 2009 in the CSA, and within each sample selected from the bi-monthly catches of the same year from the NM, are shown in Figure 5.2.

CSA

There was a significant effect of the interaction between sex and month on the copper concentration of the hepatopancreas in animals from the CSA ($F_{10,229}$=3.49, P<0.001), indicating that there was a significant difference between males and females through the year. The post hoc Tukey tests indicate that females had a higher copper concentration of the hepatopancreas than males in May (females; 285.57 ± 22.03 $\mu g.g^{-1}$: males; 157.80 ± 17.17 $\mu g.g^{-1}$), July (females; 262.25 ± 38.57 $\mu g.g^{-1}$: males; 102.92 ± 9.41 $\mu g.g^{-1}$) and August (females; 260.65 ± 22.54 $\mu g.g^{-1}$: males; 105.87 ± 18.51 $\mu g.g^{-1}$). The copper concentration of the hepatopancreas was also significantly higher in February in both males (420.34 ± 69.81 $\mu g.g^{-1}$) and females (384.51 ± 32.72 $\mu g.g^{-1}$) than other times of the year.

NM

There was no significant effect between interaction of sex and month on the copper concentration of the hepatopancreas in animals from the NM ($F_{5,104}$=1.64, P=0.158). However the effect of sex ($F_{1,104}$=28.48, P<0.001) and month ($F_{5,104}$=1.64, P<0.001) were separately significant, with females having significantly higher copper concentrations in the hepatopancreas (205.36 ± 16.29 $\mu g.g^{-1}$) than in males (132.35 ± 11.49 $\mu g.g^{-1}$). Animals in April (273.08 ± 31.12 $\mu g.g^{-1}$, both sexes combined) had significantly elevated copper concentrations of copper in the hepatopancreas than animals in any other month in 2009 (Tukey, P<0.05).

5.3.2.2 Lipid content of the hepatopancreas

The mean values (± SE) for the lipid content of the hepatopancreas in male and female *N. norvegicus,* within each sample selected from the monthly catches of 2009 in the CSA, and within each sample selected from the bi-monthly catches of the same year from the NM, are shown in Figure 5.3

CSA

There was a significant effect between the interaction of sex and month on the lipid content of the hepatopancreas in animals from the CSA ($F_{9,124}$=2.75, P=0.006), indicating that there was a significant difference between males and females through the year. The post hoc Tukey test indicated that in December 2009 females (30.56 ± 3.47%) had a significantly higher lipid content of the hepatopancreas than males (15.31 ± 2.01%).

NM

There was no significant effect between the interaction of sex and month on the copper lipid content of the hepatopancreas in animals from the NM ($F_{5,63}$=0.78, P=0.780). However the effect of sex ($F_{1,63}$=5.32, P=0.025) and month ($F_{5,63}$=15.27, P<0.001) were separately significant, with males having an annual average lipid content of 18.02 ± 1.92% and females having an average of 21.83 ± 1.80%. The post hoc Tukey test comparing the mean lipid content of the hepatopancreas in animals selected each month irrespective of sex indicates that there is a general trend of lower lipid content in the hepatopancreas in animals selected at the beginning of the year increasing throughout the year and

by the end of the year animals have significantly higher lipid levels in the hepatopancreas than at the beginning of the year.

5.3.2.3 Water content of the hepatopancreas

The mean values (± SE) for the water content of the hepatopancreas in male and female *N. norvegicus,* within each sample selected from the monthly catches of 2009 in the CSA, and within each sample selected from the bi-monthly catches of the same year from the NM, are shown in Figure 5.4.

CSA

There was no significant effect between the interaction of sex and month on the water content of the hepatopancreas in animals from the CSA ($F_{10,260}$=0.74, P=0.690). However the effect of sex ($F_{1,260}$=33.18, P<0.001) and month ($F_{10,260}$=14.11, P<0.001) were separately significant, with males having an annual average water content of 64.78 ± 0.63% and females having an annual average of 59.69 ± 0.14%. The post hoc Tukey test comparing the mean water content of the hepatopancreas in animals selected each month irrespective of sex indicates that the animals selected in the first half of the year (February to June) from the CSA have significantly higher water contents of the hepatopancreas than animals within the second half of the year (July to December).

NM

There was a significant effect between the interaction of sex and month on the water content of the hepatopancreas in animals from the NM ($F_{5,109}$=2.59, P=0.030). The post hoc Tukey test, indicated however that there was no difference between male and females in any month, which is also confirmed by the non significant effect of sex separately ($F_{1,109}$=0.03, P=0.856). The post hoc Tukey test however revealed like the animals selected from the CSA the animals selected in the first half of the year (February to June) from the NM have significantly higher water contents of the hepatopancreas than animals within the second half of the year (August to December).

5.3.2.4 Comparison between lipid and water content of the hepatopancreas

In Chapter 2 it was noted that, as the lipid content of the hepatopancreas decreases with greater nutritional stress, the amount of water will increase and the sum total of water and lipids will always be *ca* 80% of the total wet weight of the hepatopancreas. This was also the case in the present study, with 88.72% of all animals from both sites having combined lipid and water content of the hepatopancreas between 75% and 85%.

5.3.2.5 HSI

The mean values (± SE) for the HSI in male and female *N. norvegicus* within each sample selected from the monthly catches of 2009 in the CSA are shown in Figure 5.5.

CSA

There was no significant effect between the interaction of sex and month on the HSI in animals from the CSA ($F_{7,200}$=1.61, P=0.136). However the effect of sex ($F_{1,200}$=8.17, P=0.005) and month ($F_{7,200}$=5.24, P<0.001) were separately significant, with males having an annual average HSI of 6.20 ± 0.14% and females having an annual average of 5.79 ± 0.14%. The post hoc Tukey test comparing the mean HSI in animals selected each month irrespective of sex indicates that, in September, the mean HSI of both sexes is 7.35% ± 0.43, which is significantly higher than animals selected in the months or March, April, May July and August, but is not significantly higher than animals selected from the CSA in June and November.

5.3.2.6 C:N ratio of the tail muscle

The mean values (± SE) for the C:N ratio of the tail muscle in male and female *N. norvegicus,* within each sample selected from the monthly catches of 2009 in the CSA, and within each sample selected from the bi-monthly catches of the same year from the NM, are shown in Figure 5.6.

CSA

There was no significant effect between the interaction of sex and month on the C:N ratio of the tail muscle in animals from the CSA ($F_{5,113}$=2.22, P=0.058). There was also no significant effect of sex ($F_{1,113}$=0.01, P=0.939). The effect of month was however significant ($F_{5,113}$=22.69, P<0.001). The post hoc Tukey test comparing the mean C:N ratio of the tail muscle in animals selected each month irrespective of sex indicates that there is a significantly lower C:N ratio in animals selected in June in the animals sampled in the CSA (3.01 ± 0.02) compared to the highest means in August (3.23 ± 0.02).

NM

Likewise there was no significant interaction between the effects of sex and month on the C:N ratio of the tail muscle in animals from the NM ($F_{5,111}$=1.20, P=0.315). There was also no significant effect of sex ($F_{1,111}$=0.64, P=0.433). The effect of month was, however, significant ($F_{5,111}$=20.22, P<0.001). The post hoc Tukey test comparing the mean C:N ratio of the tail muscle in animals selected each month, irrespective of sex, indicates that there is a significantly lower C:N ratio in animals selected in June in the animals sampled in the NM (2.96 ± 0.01) compared to the highest means in August (3.25 ± 0.04).

Table 5.4 General linear model applied to measures made on male and females in the CSA and the NM over 2009, with the factor of Month, Sex and the interaction of Month and Sex. n= total df + 1

		copper			Lipid		
		F	df	P	F	df	P
CSA	Sex	31.69	1,229	**<0.001**	30.95	1,124	**<0.001**
	Month	13.77	10,229	**<0.001**	6.88	9,124	**<0.001**
	Sex*Month	3.49	10,229	**<0.001**	2.75	9,124	**0.006**
NM	Sex	28.48	1,104	**<0.001**	5.32	1,63	**0.025**
	Month	13.35	5,104	**<0.001**	15.27	5,63	**<0.001**
	Sex * Month	1.64	5,104	0.158	0.78	5,63	0.78

		Water			HSI		
		F	df	P	F	df	P
CSA	Sex	33.18	1,260	**<0.001**	8.17	1,200	**0.005**
	Month	14.11	10,260	**<0.001**	5.24	7,200	**<0.001**
	Sex*Month	0.74	10,260	0.690	1.61	7,200	0.136
NM	Sex	0.03	1,109	0.856			
	Month	25.9	5,109	**<0.001**			
	Sex * Month	2.59	5,109	**0.030**			

		C:N ratio		
		F	df	P
CSA	Sex	0.01	1,113	0.939
	Month	22.69	5,113	**<0.001**
	Sex * Month	2.22	5,113	0.058
NM	Sex	0.62	1,111	0.433
	Month	20.22	5,111	**<0.001**
	Sex * Month	1.20	5,111	0.315

Table 5.5 The results of the post hoc Tukey test on the significant effect of the interaction between month and sex for animals caught in the CSA for the copper concentration and the lipid content of the hepatopancreas and the NM for the water content of the hepatopancreas. Bold letters signify non-significantly different groups of means. Letters for copper and lipid values are different and cannot be cross-referred.

	CSA				NM	
	Copper		**Lipids**		Water	
	Male	**Female**	**Male**	**Female**	**Male**	**Female**
Feb-09	420.34 ± 69.81 **a**	384.51 ± 32.72 **ab**	7.50 ± 0.79 **ef**	14.57 ± 1.59 **cdef**	71.33 ± 1.43 **a**	66.56 ± 1.85 **abcd**
Mar-09	218.15 ± 20.73 **cde**	216.13 ± 15.51 **cdeg**	6.71 ± 1.01 **f**	15.27 ± 2.92 **bcdef**		
Apr-09	213.30 ± 27.02	190.15			71.93 ± 2.06 **a**	68.22 ± 1.12 **abcd**
May-09	157.80 ± 17.17 **defghi**	285.57 ± 22.03 **abc**	8.85 ± 1.48 **def**	17.55 ± 1.04 **bcdef**		
Jun-09	152.54 ± 16.54 **defghi**	241.68 ± 16.82 **cd**	14.63 ± 4.04 **bcdef**	14.00 ± 0.73 **cdef**	67.89 ± 1.14 **ab**	65.24 ± 1.13 **abcd**
Jul-09	102.92 ± 9.41 **fhi**	262.25 ± 38.57 **bcd**	15.89 ± 2.65 **bcdef**	21.91 ± 2.41 **abc**		
Aug-09	105.87 ± 18.51 **ghi**	260.65 ± 22.54 **bcd**	14.31 ± 2.55 **bcdef**	24.66 ± 2.85 **ab**	58.41 ± 2.70 **de**	59.23 ± 1.16 **bcde**
Sep-09	87.70 ± 30.26 **efghi**	175.41 ± 25.96 **cdefghi**	17.60 ± 2.14 **abcdef**	21.58 ± 1.98 **abc**		
Oct-09	80.66 ± 14.04 **i**	179.68 ± 32.79 **cdefghi**	19.36 ± 2.51 **abcd**	17.59 ± 3.27 **abcdef**	48.96 ± 2.04 **f**	57.15 ± 3.20 **cdef**
Nov-09	124.00 ± 13.77 **efgi**	147.09 ± 27.09 **defghi**	20.52 ± 3.10 **abcd**	19.57 ± 2.51 **abcde**		
Dec-09	117.73 ± 15.34 **efghi**	218.46 ± 17.00 **cdefgh**	15.31 ± 2.01 **bcdef**	30.56 ± 3.47 **a**	54.39 ± 2.14 **ef**	55.23 ± 2.36 **ef**

Table 5.6 The results of the post hoc Tukey test on the difference between month (with sex combined) for animals caught in the CSA and NM when the effect of the interaction between sex and month was not significant and the effect of month was significant. Month groups per measure that do not share a letter are significantly different. Letters within different measures are different and cannot be cross-referred.

	CSA			NM	
	Water	HSI	C:N	Copper	Lipid
Feb-09	67.14 ± 1.13 **a**		3.19 ± 0.01 **a**	157.16 ± 25.01 **bcd**	10.95 ±1.55 **c**
Mar-09	68.16 ± 0.87 **a**	5.85 ± 0.19 **b**			
Apr-09	71.69 ± 1.28 **ab**	5.35 ± 0.23 **b**	3.20 ± 0.01 **a**	273.08 ± 31.12 **a**	12.88 ± 2.32 **bc**
May-09	65.73 ± 0.99 **ab**	6.05 ± 0.38 **b**			
Jun-09	65.35 ± 0.89 **ab**	6.31 ± 0.30 **ab**	3.01 ± 0.02 **c**	204.2 ± 16.31 **b**	13.48 ± 1.53 **bc**
Jul-09	59.28 ± 1.09 **c**	5.8 ± 0.17 **b**			
Aug-09	60.62 ± 1.64 **bc**	5.46 ± 0.21 **b**	3.23 ± 0.02 **a**	164.44 ± 21.43 **bc**	19.95 ± 2.00 **b**
Sep-09	57.79 ± 1.13 **c**	7.35 ± 0.43 **a**			
Oct-09	57.12 ± 1.21 **c**		3.22 ± 0.03 **a**	82.33 ± 13.66 **d**	32.31 ± 3.45 **a**
Nov-09	60.04 ± 1.12 **c**	6.31 ± 0.23 **ab**			
Dec-09	59.56 ± 1.35 **c**		3.11 ± 0.02 **b**	119.22 ± 20.80 **cd**	29.04 ± 2.50 **a**

5.3.3 Starvation threshold

The data displayed already were then applied to the ST, as shown in Table 5.1.

5.3.3.1 Copper concentration of the hepatopancreas

The starvation threshold, as determined in Chapter 4 for the copper concentration of the hepatopancreas, is 350.19 $\mu g.g^{-1}$ for males and 283.85 $\mu g.g^{-1}$ for females, with values above these STs being indicative of starvation.

The mean values minus the SE of males from the CSA in the month of February, but for no months from the NM, were above the ST, indicative of males with a reduced nutritional status only in late winter (February) in the CSA. The mean values minus the SE of females from the CSA in the months of February and May, and only for the month of April from the NM, were above the ST, indicative of females with a reduced nutritional status during late winter and spring.

5.3.3.2 Lipid content of the hepatopancreas

The starvation threshold, as determined in Chapter 4 for the lipid content of the hepatopancreas is 8.30% for males and 26.83% for females, with values below these ST being indicative of starvation.

The mean values plus the SE of males from the CSA in the months of February and March, but for no months from the NM, were below the ST, indicative of males with a reduced nutritional status only in late winter, and only in the CSA. The mean values minus the SE of females from the CSA in the month of February and from the NM in the months April, June and August were below the ST, indicative of females with a reduced nutritional status in late winter in the CSA, but across the spring and summer in the NM.

5.3.3.3 Water content of the hepatopancreas

The starvation threshold, as determined in Chapter 4, for the water content of the hepatopancreas is 68.64% for males and 58.33% for females, with values above these ST being indicative of starvation.

The mean values minus the SE of males from the CSA in the months of February-May and for males from the NM in the months February and April were above the ST, indicative of males with a reduced nutritional status in the spring at both

sites. The mean values minus the SE of females from the CSA in the months of February- May and of females from the NM in the months February, April, June and August were above the ST, indicative of females with a reduced nutritional status in the spring in the CSA, and over the spring and summer in the NM.

5.3.3.4 HSI

The starvation threshold, as determined in Chapter 4, for the HSI for males is 3.44% and for females is 4.69%, with values below these ST being indicative of starvation.

The mean values plus the SE of males and females from the CSA were greater than this ST on all occasions, indicative of no reduction in the nutritional status of *N. norvegicus* of either sex captured from the CSA throughout the year.

5.3.3.5 C:N ratio of the tail muscle

The starvation threshold, as determined in Chapter 4, for the C:N ratio of the tail muscle for males is 3.07, but was not determined for females, with values below this ST being indicative of starvation.

The mean values plus the SE of males from the CSA and NM in the month of June were below the ST, indicative of animals with a reduced nutritional status in this summer month.

Table 5.7 Summary of indications of nutritional status given by the mean values of each biochemical measure in relation to the derived starvation thresholds data for each month of the year for the CSA and NM. '*'- indicates reduced nutritional status; 'blank' - indicates no reduced nutritional status; '?'- indicates a mean which is close to the ST. '-' indicates not measured.

		Male					Female				
		Copper	**Lipid**	**Water**	**HSI**	**C:N ratio**	**Copper**	**Lipid**	**Water**	**HSI**	**C:N ratio**
CSA	Feb-09	*	*	*	-		*	*	*	-	-
	Mar-09			*					*		-
	Apr-09			*					*	?	-
	May-09			*			*		*		-
	Jun-09			?		?			*		-
	Jul-09										-
	Aug-09										-
	Sep-09										-
	Oct-09				-					-	-
	Nov-09										-
	Dec-09				-					-	-
NM	Feb-09			*	-				*	-	-
	Apr-09			*	-		*	*	*	-	-
	Jun-09			*	-	*		*	*	-	-
	Aug-09				-			*	*	-	-
	Oct-09				-					-	-
	Dec-09				-					-	-

5.3.4 Site and month interaction

The difference in means between sites was considered. Tests of the interaction between the effects of site and month are considered in males and females separately for each measurement. As the NM was only sampled bi-monthly, the animals from the CSA were also analysed bi-monthly to serve as a direct comparison. The statistical results of these tests can be seen in Table 5.8.

5.3.4.1 Copper concentration of the hepatopancreas

Males

There was a significant interaction between the effects of site and month on the copper concentration of the hepatopancreas in males ($F_{5,118}$=10.14, P<0.001), indicating that the sites differed. The post hoc Tukey test indicates that in February, male *N. norvegicus* from the CSA (420.34 ± 69.81 $\mu g.g^{-1}$) had a significantly higher copper concentration of the hepatopancreas than males in

the NM (143.27 ± 36.10 $\mu g.g^{-1}$) of the same month. In other months, this measure did not differ significantly between the two sites.

Females

There was a significant interaction between the effects of site and month on the hepatopancreas copper concentration of females ($F=_{5,100}$=3.67, P=0.005). The post hoc Tukey test indicates that, as with males, female *N. norvegicus* in February from the CSA (384.51 ± 32.72 $\mu g.g^{-1}$) have a significantly higher copper concentration of the hepatopancreas than males in the NM (184.94 ± 20.39 $\mu g.g^{-1}$), with measures from other months not differing significantly between the two sites.

5.3.4.2 Lipid content of the hepatopancreas

Males

There was a significant interaction between the effects of site and month on the lipid content of the hepatopancreas in males ($F_{4,59}$=3.98, P=0.007), indicating that the sites differed. The post hoc Tukey test indicate that in October, male *N. norvegicus* from the CSA (19.36 ± 2.51%) had a significantly lower lipid content of the hepatopancreas than males in the NM (33.21 ± 3.43%) of the same month. In other months, this measure did not differ significantly between the two sites.

Females

There was no significant interaction between the effects of site and month on the lipid content of the hepatopancreas in females ($F_{4,62}$=2.19, P=0.083), indicating that sites did not differ.

5.3.4.3 Water content of the hepatopancreas

Males

There was a significant interaction between the effects of site and month on the water content of the hepatopancreas in males ($F_{5,124}$=2.59, P=0.029), indicating that the sites differed. The post hoc Tukey test indicate that in October, male *N. norvegicus* from the CSA (58.66 ± 1.71%) had a significantly higher water content of the hepatopancreas than males in the NM (48.96 ± 2.04%) of the same

month. In other months, this measure did not differ significantly between the two sites.

Females

There was no significant interaction between the effects of site and month on the water content of the hepatopancreas in females ($F_{5,118}$=0.48, P=0.825), indicating that sites did not differ.

5.3.4.4 C:N ratio of the tail muscle

Males

There was no significant interaction between the effects of site and month on the C:N ratio of the tail muscle in males ($F_{5,117}$=1.24, P=0.295), indicating that sites did not differ.

Females

There was a significant interaction between the effects of site and month on the C:N ratio of the tail muscle in females ($F_{5,106}$=6.43, P<0.001), indicating that the sites differed. The post hoc Tukey test indicate that in June, female *N. norvegicus* from the CSA (3.05 ± 0.01) had a significantly higher C:N ratio of the tail muscle than females in the NM (2.96 ± 0.02) of the same month; and in December, female *N. norvegicus* from the CSA (3.08 ± 0.03) had a significantly lower C:N ratio of the tail muscle than females in the NM (3.21 ± 0.03) of the same month. In other months, this measure did not differ significantly between the two sites.

Table 5.8 GLM applied to measures made on male and females between the interaction between the effects of site and month. Significant P values (P<0.05) in bold. n= total df + 1

		Copper			Lipid		
		F	df	P	F	df	P
Male	Site	13.34	1,118	**<0.001**	7.8	1,59	**0.007**
	Month	21.95	5,118	**<0.001**	14.64	4,59	**<0.001**
	Site * Month	10.14	5,118	**<0.001**	3.98	4,59	**0.007**
Female	Site	1.84	1,100	0.178	2.56	1,62	0.116
	Month	5.4	5,100	**<0.001**	13.55	4,62	**<0.001**
	Site * Month	3.67	5,100	**0.005**	2.19	4,62	0.083

		Water			C:N ratio		
		F	df	P	F	df	P
Male	Site	8.87	1,124	**0.004**	1.95	1,117	0.166
	Month	24.23	5,124	**<0.001**	26.29	5,117	**<0.001**
	Site * Month	2.59	5,124	**0.029**	1.24	5,117	0.295
Female	Site	0.20	1,118	0.654	2.08	1,106	0.153
	Month	12.74	5,118	**<0.001**	22.32	5,106	**<0.001**
	Site * Month	0.43	5,118	0.825	6.43	5,106	**<0.001**

5.3.4.5 Size differences between the sexes and sites within months

Utilising the composition samples, the mean size (as CL) of males and females selected from the CSA (north transect) were 33.84 ± 0.42 mm and 34.14 ± 0.42 mm, respectively. There was a significant effect of the interaction between sex and month ($F_{5,1046}$=15.78, P<0.001). The post hoc Tukey test results (Table 5.9) indicate that males caught in the CSA were significantly larger than females in all but the June and August samples.

The mean sizes (as CL) of males and females selected from the NM transect were 31.96 mm ± 0.16 and 27.84 mm ± 0.145, respectively. There was a significant effect of the interaction between sex and month ($F_{5,1641}$=12.34, P<0.001). The post hoc Tukey test result (Table 5.10) indicates that males caught from the NM were significantly larger than females in all but the August sample, coinciding with peak emergence of females.

There was no significant effect of the interaction between site and month in the mean sizes between sites of males ($F_{3,844}$=2.09, P=0.100), the effect of this interaction in females was, however, significant with animals from the CSA in

June (30.95 ± 0.37 mm) and August (32.22 ± 0.66 mm) being significantly larger than females from the NM in the same months (June; 28.21± 0.21 : August; 29.32 ± 0.30) (Tukey, P=0.05).

Table 5.9 The body sizes (as mean ± SE CL) of male and female *N. norvegicus* from the CSA and the NM throughout 2009. Letters represent separate post hoc Tukey pairwise comparison tests for each site. Means that do not share a letter are significantly different. N=1047

	CSA		NM	
	Male	**Female**	**Male**	**Female**
Feb-09	31.44 ± 0.49 **b c d**	26.52 ± 0.62 **e f**	30.58 ± 0.34 **c d**	24.38 ± 0.28 **e f**
Apr-09	32.23 ± 0.67 **b c**	29.20 ± 0.45 **d f**	34.20 ± 0.31 **a**	29.25 ± 0.56 **c d e f**
Jun-09	29.91 ± 0.61 **c d**	30.95 ± 0.37 **b c d**	30.57 ± 0.52 **c d**	28.21 ± 0.21 **e f**
Aug-09	31.82 ± 0.53 **b c d**	32.22 ± 0.66 **b c**	30.08 ± 0.47 **c d**	29.32 ± 0.30 **d e**
Oct-09	35.46 ± 0.83 **a**	29.47 ±1.24 **b c d e f**	32.92 ± 0.51 **a b**	27.00 ± 0.41 **e f g**
Dec-09	32.01 ± 0.31 **b**	26.05 ± 0.45 **f**	31.39 ± 0.32 **b c**	26.40 ± 0.29 **f g**

Expressing these data for both sexes and both sites in size categories (10-20, 20-30, 30-40, 40-50, and 50+ mm CL) there is a distinct sex difference in the seasonal fluctuation of body size of the animals both in the CSA (Figure 5.7) and the NM (Figure 5.8). At both sites the size distribution of the males fluctuated throughout the year, but with no overall seasonal pattern, whereas the females caught in the winter were mainly less than 30 mm CL, while in the summer there was a greater proportion in the 30-40 mm CL category, with the appearance of the largest females only in summer, smallest males in spring, and smallest females in late winter/early spring.

5.3.5 Effects of the reproductive condition of females on their nutritional status

From the field data analysed above, females would appear to be at a higher nutritional status than males. The next stage was to establish whether females have a lower nutritional status than males at the beginning of the year, or if this difference is due to a sex-specific physiology.

To determine whether any of the females that were available for capture throughout the winter were actually immature, the size at onset of maturity (SOM) was calculated. The SOM was measured in two alternative ways, namely the size of which 50% of the females had matured ovaries (L_{50}) and the smallest size of ovigerous females (see Chapter 1). In the CSA the L_{50} was determined to be 27 mm CL and the smallest ovigerous female found was 26 mm; in the NM the L_{50} was determined to be 27 mm and the smallest ovigerous female found was 25 mm. In each case the smaller of the two measures was taken as a conservative estimate of SOM, to ensure only immature females were classed as such. The white lines on Figure 5.7b and Figure 5.8b represent the SOM. They indicate that not all females in the size category 20-30mm CL were immature. However, a greater proportion of those in the catches in February and December were immature females, when compared with the females caught in July.

A separate analysis was carried out on the 450 females obtained in the CSA in June and December 2010 for determining sexual maturity. HSI and gonad staging were determined for each of these animals as described in Chapter 1 and shown in Table 1.1. Females from the whole catch were grouped into three broad categories: large, medium and small, and at least 100 individuals from each of the large and small categories were measured.

There was a significant difference in terms of the HSI between females measured in June and in December (June 7.47 ± 0.08%, December 8.61 ± 0.20%; $F_{1,449}$=35.70, P<0.001). However, there was no significant difference in the HSI between females larger than 26 mm (SOM) compared with those smaller than 26 mm ($F_{1,449}$=2.49, P=0.115), indicating that there was no difference in nutritional status between mature and immature females sampled.

5.3.5.1 Effect of egg presence on nutritional status

As the processed catches (used to determine nutritional status) targeted larger animals, immature females were not retained in significant numbers. Therefore comparisons in relation to maturity were not possible. All subsequent analyses were therefore carried out only on mature females (i.e. above the SOM).

A comparison of the biochemical parameters between ovigerous and non-ovigerous (but mature) female *N. norvegicus* was carried out in order to determine if they were affected by the egg-bearing condition; the mean values of each measure between ovigerous and non-ovigerous females are shown in Table 5.10.

As there was no major difference in the post hoc Tukey results (Table 5.6) in the HSI or in the tail muscle C:N ratio measurements in females, no further analysis was carried out on these parameters.

Copper concentration of the hepatopancreas

The mean values (± SE) for the copper concentration of the hepatopancreas between ovigerous and non-ovigerous *N. norvegicus* selected from the monthly catches in 2009 in the CSA, and from the bi-monthly samples of the same year in the NM are shown in Figure 5.9.

There was no significant difference in the copper concentration of the hepatopancreas between ovigerous (292.46 ± 33.60 $\mu g.g^{-1}$) and non-ovigerous (264.66 ± 14.98 $\mu g.g^{-1}$) females in the CSA ($F_{1,42}$=0.49, P=0.487) nor between ovigerous (213.96 ± 24.30 $\mu g.g^{-1}$) and non-ovigerous (198.13 ± 22.30 $\mu g.g^{-1}$) females in the NM ($F_{1,43}$=0.29, P=0.596).

Lipid content of the hepatopancreas

The mean values (± SE) for the lipid content of the hepatopancreas between ovigerous and non-ovigerous *N. norvegicus* selected from the monthly catches in 2009 in the CSA, and from the bi-monthly samples of the same year in the NM are shown in Figure 5.10. There was no significant difference in the lipid content of the hepatopancreas between ovigerous (18.36 ± 1.03%) and non-ovigerous (14.67 ± 0.87%) females in the CSA ($F_{1,31}$=3.77, P=0.062) nor between ovigerous

(23.19 ± 2.55%) and non-ovigerous (19.26 ± 1.83%) females in the NM ($F_{1,25}$=0.85, P=0.367).

Hepatopancreas water

The mean values (± SE) for the water content of the hepatopancreas between ovigerous and non-ovigerous *N. norvegicus* selected from the monthly catches in 2009 in the CSA (a), and in the bi-monthly samples of the same year in the NM (b), are shown in Figure 5.11. There was a significant difference in the water content of the hepatopancreas between ovigerous and non-ovigerous females in the CSA ($F_{1,50}$=6.42, P=0.015), with ovigerous females having a lower mean water content (60.62 ± 0.83%) compared with non-ovigerous females (64.51 ± 0.59%). However, in the NM there was no significant difference in this measure between ovigerous (58.83 ± 1.85%) and non-ovigerous (63.23 ± 1.03%) females ($F_{1,49}$=2.92, P=0.094).

Table 5.10 the GLM results and the Mean ± SE of copper concentration, lipid and water content and of the hepatopancreas of ovigerous and non-ovigerous female *N. norvegicus*. Significant difference (P<0.05) in bold. n= total df + 1

		F	df	P	Ovigerous	Non-ovigerous
CSA	Copper ($\mu g.g^{-1}$ wet)	0.49	1,42	0.487	292.46 ± 33.60	264.66 ± 14.98
	Lipid (% wet wt)	3.77	1,31	0.062	18.36 ± 1.03	14.67 ± 0.87
	Water (% wt)	6.42	1,50	**0.015**	60.62 ± 0.83	64.51 ± 0.59
NM	Copper ($\mu g.g^{-1}$ wet)	0.29	1,43	0.596	213.96 ± 24.30	198.13 ± 22.30
	Lipid (% wet wt)	0.85	1,25	0.367	23.19 ± 2.55	19.26 ± 1.83
	Water (% wt)	2.92	1,49	0.094	58.83 ± 1.85	63.23 ± 1.03

5.3.5.2 Effect of gonad stage on female biochemistry

The proportion of females in each stage of gonadal maturation in the female portion of the sub-samples in the monthly catches in the CSA and the bi-monthly samples in the NM are shown in Figure 5.12. The females caught in the winter months had less-well developed ovaries than those in the summer. A progression

in the maturation cycle can be seen within the first half of the year, until by July (in CSA) and June (in NM) all females sampled had ovaries developed to stage 3 or above. In the second half of the year an increased proportion of females had spent ovaries due to them having spawned recently. The mean (± SE) GSI in these samples of females from the CSA also increased from March to July (Figure 5.13), then decreased after an increasing proportion of females had spawned.

In order to determine the nutritional status of females in relation to seasonal changes in ovary condition (Table 5.12) the data from the catches for biochemical analyses were analysed in relation to the ovary stages of these animals. To yield sufficient numbers in the developmental categories, the 8 stages were amalgamated into three groups (A, stages 1-2 and 8; B, stages 3-4; and C, stages 5-7). The means (± SE) of each measure in animals in all three ovary maturation categories are shown in Table 5.11.

Table 5.11 The numbers of females in different stages of ovary maturation within the processed catch

Site	Clyde Sea Area										North Minch								
Group	A		B		C			A			A		B		C			A	
Ovary stage	1	2	3	4	5	6	7	8	Totals		1	2	3	4	5	6	7	8	Totals
Feb-09	4	1	0	4	1	0	0	3	**13**		2	0	0	0	2	1	0	0	**5**
Mar-09	3	2	0	3	0	0	5	0	**13**										
Apr-09	1	1	0	2	7	0	0	0	**11**		1	0	0	5	1	0	1	0	**8**
May-09	1	0	2	4	4	0	0	0	**11**										
Jun-09	0	0	0	1	12	0	0	0	**13**		0	0	1	3	1	7	0	0	**12**
Jul-09	0	0	0	0	13	0	0	2	**15**										
Aug-09	0	0	0	0	5	1	1	6	**13**		0	0	0	0	8	-	-	7	**15**
Sep-09	0	0	0	0	8	0	0	0	**8**										
Oct-09	2	1	1	0	0	1	4	2	**11**		8	0	0	0	0	0	0	2	**10**
Nov-09	4	3	1	2	1	0	0	1	**12**										
Dec-09	5	0	0	1	0	0	0	8	**14**		6	6	0	0	0	0	0	3	**15**

Copper concentration of the hepatopancreas

The mean values (± SE) for the copper concentration of the hepatopancreas in relation to the stage of ovary development in female *N. norvegicus* sampled within the first half of 2009 in the CSA and the NM are shown in Figure 5.14. There was no significant difference in this measure amongst females at different

stages of ovary development in either the CSA ($F_{2,42}$=1.42, P=0.255) or the NM ($F_{2,16}$=0.10, P=0.908) as shown in Table 5.12.

Lipid content of the hepatopancreas

The mean values (± SE) for the lipid content of the hepatopancreas in relation to the stage of ovary development in female *N. norvegicus* sampled within the first half of 2009 in the CSA and the NM are shown in Figure 5.15. There was no significant difference in this measure amongst females at different stages of ovary development in the CSA ($F_{2,31}$=1.44, P=0.253) or in the NM ($F_{2,19}$=2.45, P=0.156) as shown in Table 5.12.

Water content of the hepatopancreas

The mean values (± SE) for the water content of the hepatopancreas in relation to the stage of ovary development in female *N. norvegicus* sampled in the first half of 2009 in the CSA and the NM are shown in Figure 5.16. There was a significant difference in this measure amongst females at different stages of ovary development in the CSA ($F_{1,50}$=6.42, P=0.015) with females with white, cream or spent ovaries (group A) having a mean water content of 66.13 ± 1.04%, females with very pale and pale green ovaries (group B) having a mean water content of 62.48 ± 1.23% and females with green- to dark green ovaries (group C) having a mean water content of 63.46 ± 0.62%. There was, however, no significant difference in this measure amongst females at the different stages of ovary development in the NM ($F_{2,18}$=0.92, P=0.417) as shown in Table 5.12.

Table 5.12 The Mean ± SE of water and lipid content and copper concentration of the hepatopancreas of female *N. norvegicus* in three different ovary maturation stage groups A (white and cream), B (pale green to green) and C (dark green). n= total df + 1

		F	df	P	**A**	**B**	**C**
CSA	Copper ($\mu g.g^{-1}$ wet)	1.42	2,42	0.255	15.90 ± 2.04	16.91 ± 1.47	13.95 ± 0.79
	Lipid (% wet wt)	1.44	2,31	0.253	266.46 ± 33.54	305.42 ± 30.43	249.50 ± 12.99
	Water (% wt)	3.79	2,50	**0.03**	66.13 ± 1.04	62.48 ± 1.23	63.46 ± 0.62
NM	Copper ($\mu g.g^{-1}$ wet)	0.1	2,16	0.908	183.75 ± 30.40	327.62 ± 68.84	206.55 ± 19.00
	Lipid (% wet wt)	2.45	2,9	0.156	25.45 ± 5.74	14.45 ± 1.48	19.88 ± 1.65
	Water (% wt)	0.92	2,18	0.417	58.00 ± 2.15	66.45 ± 1.68	63.48 ± 1.23

5.4 Discussion

The results presented in this chapter clearly indicate that the nutritional status of *N. norvegicus* varies with a number of factors, including season, site, sex and the reproductive condition of the female. On the basis of these results, a number of conclusions can be drawn about the feeding behaviour of this species at different times of year, and, when combined with the biometric data obtained, inferences can also be made about the emergence behaviour of females over the winter period.

5.4.1 Seasonal interactions

There was significant variation in all parameters measured in animals throughout the year. The results obtained from animals caught in the first half of the year indicated that during this period animals may have a reduced nutritional status compared with the second half of the year. This was indicated by the hepatopancreas water, lipids and copper measurements, as well as the tail muscle C:N ratio (in males only). This is consistent with similar results from quarterly samples of *N. norvegicus* taken in 1992-93 by IMBC *et al.* (1994) and bimonthly samples in 1995-96 by Parslow-Williams (1998). They showed that the

lowest lipid and highest water in the hepatopancreas occurred in the spring, and the inverse for the summer. However, HSI did not vary significantly between these two seasons.

Why would animals have a lower nutritional status in the winter than the summer? There are two possible explanations, firstly that there is less food available, and secondly that there is less consumption of the food that is present. As explained in the introduction, food available in the benthos comes from the water column above, and in the winter months there is a reduction in primary production in the water column and thus the amount being driven down to the benthos. This theory was also suggested by IMBC *et al.* (1994).

Less consumption of any food that is present is also possible during the winter due to a reduced burrow emergence. This might be as a response to shorter day lengths, lower light levels, greater cloud cover or water turbulence due to bad weather, causing *N. norvegicus* to remain in their burrows for more extended periods of time in the winter months (Maynou & Sarda, 2001). The starvation threshold (ST) classifications of each group of *N. norvegicus* throughout the year shown in Table 5.7 support the idea that the individuals caught at the beginning of the year had a lower nutritional status than those taken at the end of the year.

A detailed analysis of catch data, resolved by area and month, may give further clues to the nature of foraging behaviour during the winter and reveal if there are seasonal differences in such behaviour, but this was beyond the scope of the present investigation.

5.4.1.1 Copper concentration of the hepatopancreas

There were noticeable peaks in the copper concentration of the hepatopancreas in both male and female animals in February in the CSA and in April in the NM, which were over the ST. At the same time the water, lipid and HSI measurements also indicated a reduced nutritional status (however were not all over the ST). Why could this be the case?

As mentioned in Chapter 2 the copper concentration of the hepatopancreas is linked with the copper concentration of the haemocyanin within the haemolymph. If the haemocyanin concentration decreases and if Depledge & Bjerregaard (1989) are correct in suggesting the hepatopancreas is the principal site of copper storage, then the copper concentration in the hepatopancreas will rise.

As both males and females had a copper concentration in the hepatopancreas that exceeded the ST in February, then the peak in hepatopancreas copper cannot be explained by physiological characteristics particular to females. Taylor & Anstiss (1999) suggested six different factors which can affect the concentration of haemocyanin, and thus potentially the concentration of copper, in the hepatopancreas. These are: haemorrhage, parasitism, moulting, salinity change, hypoxia or starvation. The first of these can be discounted immediately as haemorrhaging would be on an individual bases and would not affect all the animals which were caught in February. The others are considered in more detail below.

Moulting

As stated in Chapter 4 (Section 4.4.2.1), the period around the moulting event is a period of substantial physiological change in *N. norvegicus* and thus could possibly be an influence on the copper concentrations measured in this study. The cessation of oxygen consumption by the animal during ecdysis may reduce the amount of haemocyanin in postmoult animals, leading to an increase in the copper concentration in the hepatopancreas. In the present study, the animals measured were selected to be in the intermoult state (i.e. with hard carapaces), and so processes associated with ecdysis are unlikely to explain the observed peak in hepatopancreas copper. Also a synchronous moult at this time is seen only in females, and any moult-related effect would therefore be expected to be seen only in females, whereas in fact the peak in hepatopancreas copper levels occurred in both sexes.

Parasitism

Also as noted in Chapter 4 (Section 4.4.2.2), although the parasitism of *N. norvegicus* with *Hematodinium* sp. is known to increase oxygen consumption (Taylor *et al.*, 1996), the animal will still accumulate copper in the

hepatopancreas. Field *et al.* (1992) and Beevers *et al.* (2012) showed that the peak period of the prevalence of this parasite was in the months February to May; this may be what is being picked up in these animals. Animals were not tested for *Hemtodinium* sp. infection; however, no obvious sign of infection was observed. Beevers *et al.* (2011) stated that when animals were over 30 mm CL their susceptibility to infection was reduced. Animals caught in February were 32.20 ± 0.89 mm CL in males and 30.35 ± 1.94 mm CL in females and therefore the peak in hepatopancreas copper is unlikely to be the effect of parasitism by *Hematodinium* sp., but cannot be fully ruled out.

Salinity

Low salinities can cause toxic metal absorption from the environment, changes that are not linked to the use of copper by the organism for normal biological purposes. Rippeth & Simpson (1983) showed that there were weak seasonal cycles of salinity at all depths in the CSA, due to fluctuating fresh water inputs, causing salinity to be lower in winter and spring. If this was a significant factor, the expectation would be for copper to decrease in the water, and hence in the body tissues, at these times of reduced salinity, but in fact the results obtained indicate a sharp increase instead. An explanation based on salinity changes therefore seems very unlikely.

Hypoxia

Baden *et al*. (1990) showed the effect of hypoxia (an oxygen concentration reduced by at least 2 $ml.L^{-1}$) on the blood physiology of *N. norvegicus*, leading to a loss of circulating haemocyanin. A cessation of feeding is also thought to occur under these conditions. Periods of hypoxia could therefore add to the bioaccumulation of copper in the hepatopancreas. Bell *et al*. (2006) noted that *N. norvegicus* are occasionally observed in areas of severe hypoxia, which cause mass mortalities, or in areas with less severe hypoxia, which lead to *N. norvegicus* being out of the burrows for more prolonged periods, making them more vulnerable to trawling. Thus, an explanation of the redistribution of copper from haemocyanin in the blood to the hepatopancreas being a response to exposure to hypoxia would predict that this would be accompanied by an increase in the number of females encountered in trawl catches, which the results indicate is not in fact the case. An explanation based on a response to hypoxia therefore seems unlikely.

Starvation

Finally, Taylor & Anstis (1999) suggested that starvation is a factor in the reduction of circulating haemocyanin. It was shown in Chapter 2 that under starvation conditions the haemolymph copper concentration decreased, and it was concluded that as haemocyanin is the major copper-based protein in the haemolymph then it must be closely linked. There was also a strong correlation under greater food limitation between the decreasing amount of copper in the haemolymph and the increasing amount in the hepatopancreas. For this reason the copper concentration of the hepatopancreas was used in this study. This measure provides the first indication of lowered nutritional status, and therefore it could be the case that these animals were nutritionally stressed for around 12 weeks (in line with findings in Chapter 2). It is therefore possible that what we are seeing is related to lack of food in the winter period. This is also supported by other measures reported in this chapter.

It is difficult to conclude definitively which of these effects account for the spike in copper in late winter. It seems that a combination of parasitism and starvation is the most likely.

5.4.1.2 C:N ratio of the tail muscle

The C:N ratio of the tail muscle was lower in both males and females from both sites in the month of June, suggesting that the biochemistry of the tail muscle has altered or the animals available for capture were different in June 2009; starvation is unlikely to be the reason for this as no other measurements have indicated it within this month. Future investigation would be needed to see if this was just a phenomenon that occurred within both sites at the same time or if this is an annual phenomenon.

5.4.2 Site comparison

In many of the months there was no significant difference between males or females comparing between the CSA and the NM. When there was significant difference in individual months the animals from the NM had a greater nutritional status than those of the CSA. This is consistent with the hypothesis that the density of *N. norvegicus* is correlated with their nutritional status, as

stated by Tuck *et al.* (1997c) and Parslow-Williams (1998), since burrow density was higher in the CSA (ICES, 2011). Consideration of other possible factors that might contribute to site differences will be postponed until after the results of studies on the trophic dynamics at these sites are reported in the following two chapters (Chapters 6 and 7).

5.4.3 Sex specific interactions

A consistent finding was that the hepatopancreas-based parameters were significantly different between the two sexes. This was the case for all these parameters in the animals from the CSA, and in all but the water content of the hepatopancreas in the NM. IMBC *et al.* (1994) also showed that females generally have higher lipid and lower water content than males; they also found that females have lower protein levels in the hepatopancreas than males (a measure not made in this study). The starvation trials in the present study (Chapter 3) showed that females have higher lipid and significantly lower water content of the hepatopancreas than do males treated in a similar way.

Females with white (Stage 1) ovaries have significantly higher water content within the hepatopancreas than females with later staged ovaries. Rosa & Nunes (2002b) showed that both cholesterol and therefore total lipids increased in the hepatopancreas at the later stages of ovary development. They then significantly decreased from around 41% lipid dry weight to 29% in the first stages of ovary development (when they are still white – Stage 1). This fall in lipid content is accompanied by a compensatory increase in water content (seen in section 5.2.1.2). This is more probably due to the changing mass of the ovaries, reflected in the GSI being *ca* 8% when the ovaries are mature, but only *ca* 1% when they are spent, rather than being related directly to feeding rates.

5.4.3.1 Do females feed over the winter?

It is now appropriate to consider the nutritional strategy of females over the winter brooding period, and to evaluate the evidence for and against the possibility that females do not feed over this period.

Burrow-related behaviour of females during the winter

The sex ratios recorded monthly through the year indicate a pattern of increasing emergence of females from their burrows during the spring. Over the winter period, however, females are far less abundant in trawl catches, consistent with them spending much of their time within their burrow systems, as has also been shown in many previous studies (Oakley, 1978; Bailey, 1984; Briggs, 1995; Tuck *et al.* 1997a; Milligan *et al.* 2009).

Aguzzi *et al.* (2007) hypothesised that females do not remain completely in their burrows, but rather change the range and duration of their excursions. Main & Sangester (1985) showed that animals that were seen in close proximity to their burrows were able to escape quickly from predators or fishing nets. The occurrence of ovigerous females in creels in winter also suggests opportunistic feeding emergence (Adey, 2007).

Oakley (1978) observed that in the winter months of a controlled experiment under artificial conditions (November-May) ovigerous females rarely came out beyond 25 cm from the burrow entrance to collect food that had been detected. They were also observed to carry large food items into burrow systems for consumption. Females kept for an extended period of time in a mesocosm have shown 'caching' behaviour by burying food items away from the burrow, presumably so as not to attract predators (P. Cowie pers. comm.) it is also known that *N. norvegicus* will also bury food within their burrow. This suggests that females could in fact 'prepare' for the winter months by creating a readily accessible food store.

Of the small numbers of females caught during the winter a few were mature (and in rare cases ovigerous), but the mean size of winter females was smaller than at other times of year, indicating a high percentage of smaller females that were either immature or within their first reproductive cycle. Relevant to this finding, Thomas & Figueiredo (1965) stated that immature sizes of female *N. norvegicus* show no seasonal variation in abundance.

The feeding strategy adopted by females during the winter

In the present study there was a difference in females compared with males in terms of the indicators of nutritional status. There are two possible explanations

for such differences between sexes. Firstly it may be a sex-specific physiological difference related to the female reproductive cycle, or secondly it may be due to feeding differences between the sexes.

There was no evidence obtained in this study to suggest that females ever utilised coping mechanisms other than metabolic depression over the winter months, suggesting that they probably did obtain food over the winter, rather than call extensively on internal reserves. Females have been reported to be caught in baited creels throughout the year (Adey, 2007) suggesting that they can be tempted out of the burrows when they detect food. Due to the significantly higher concentration of copper in female *N. norvegicus* in the CSA when compared to males from May to August this would suggest that the metabolism of females may have been depressed further than that of males, even in the spring and early summer months. This may be due to a reduced amount of food or merely that they are in a more sedentary state due to the greater amount of time they spend within their burrows. This is true in winter, but probably not in May to August, when females seem to be more catchable than males, as judged by the sex ratio of the catch. They are thought to feed intensively before the next period of egg bearing (as evidenced by increase in lipid in hepatopancreas and ovaries over summer). This would need to be tested further but could suggest that the feeding rate could acutely be lower in females than males, throughout the year.

This interpretation is in line with the results of Aguzzi *et al.* (2007) who showed that there was a significantly higher percentage of ovigerous females with empty stomachs, compared to non-ovigerous females and to males. They concluded that, due to the enlarged size of the ovaries, there is less space in the cephalothorax of ovigerous females for the stomach to fill with food, and this could lead to hunger suppression. This suggests that females could be feeding at a reduced rate over the winter when compared with males, and indicates that ovigerous females will not be out on the bottom sediment away from their burrows when bearing eggs. In fact Newland (1985) showed that ovigerous females have a reduced reaction to an external stimulus compared to non-ovigerous females or males, and that this leads to the tail flipping escape behaviour being reduced to retain eggs on the pleopods.

The need for continued feeding over the winter

As the development of the female ovary is one of the most significant metabolic events in the physiology of crustaceans (Rosa & Nunes, 2002a), and since the ovaries develop over the whole year, even while the female is brooding the eggs from the previous reproductive cycle (as seen in this study and Bailey, 1984), it is likely that females will need to continue feeding throughout the year.

Rosa & Nunes (2002a) showed that there is no significant decrease in the lipid content of the hepatopancreas in females during development of the ovaries. Also, Rosa & Nunes (2002a) and Tuck *et al.* (1997a) showed that the HSI remains stable or even increases throughout the development of the ovary. These findings indicate that resources are not being depleted from the hepatopancreas to supply the developing ovaries. More particularly, steroid hormones are required to develop the female gonad (Rosa *et al.* 2003), and these are derived from cholesterol, which cannot be produced by *de novo* synthesis but needs to be acquired through the diet. Therefore it seems that females must continue to acquire food throughout the developmental cycle of the ovaries, in order to support their maturation. Indeed, Talbot & Helluy (1995) suggest that in the lobster *Homarus americanus* reproductive success is negatively influenced by a shortage of food.

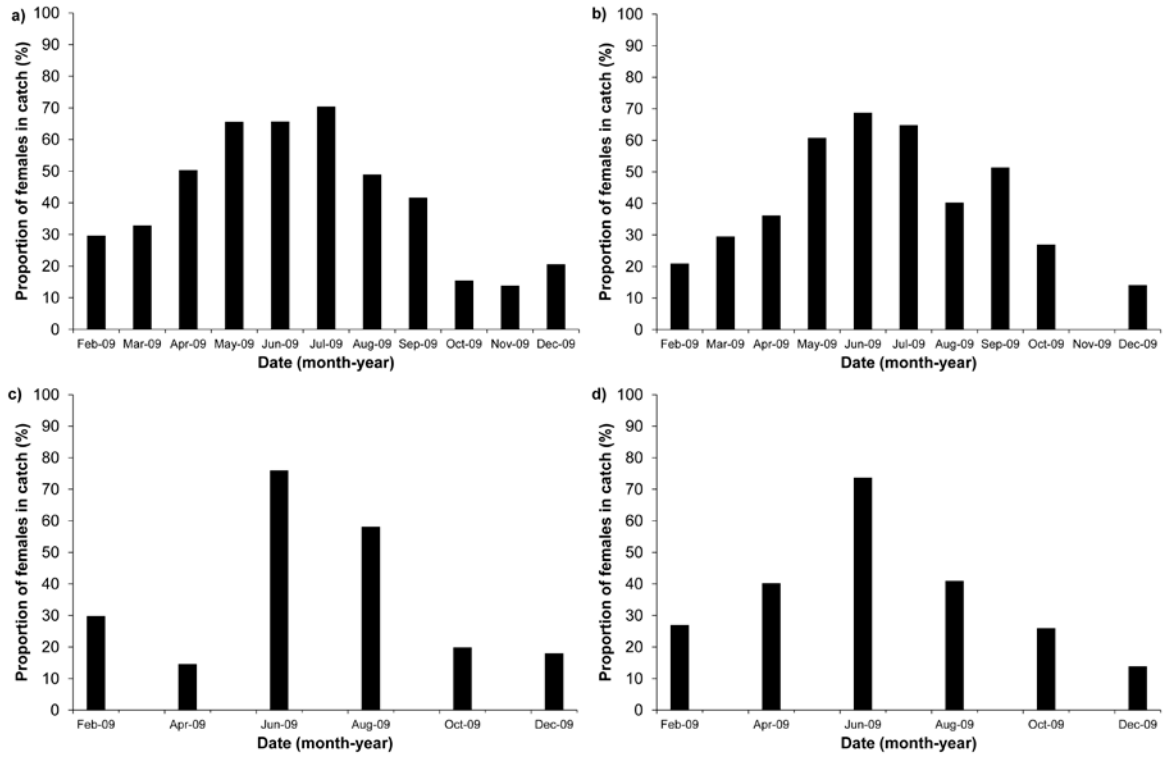

Figure 5.1 Proportion of the representative catch (sex ratio) made up of female *N. norvegicus* from the Clyde Sea Area a) north and b) west transect and the North Minch c) east and d) south transects.

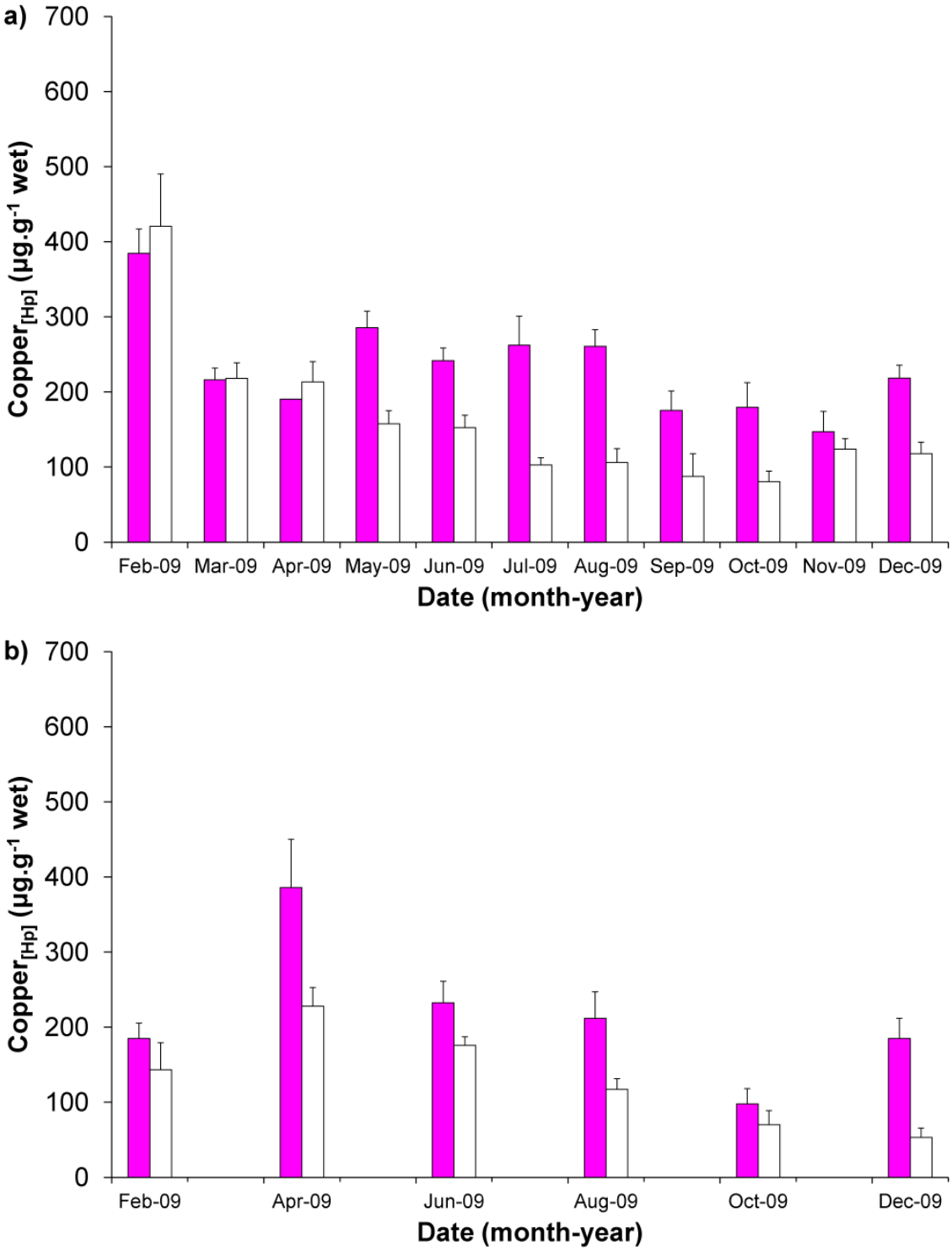

Figure 5.2 Copper concentration of the hepatopancreas (means ± SE) in female (pink bars) and male (white bars) groups of *N. norvegicus* caught on the a) Clyde Sea Area- North Transect and b) North Minch- East transect throughout 2009. a) n=230, b) n=105

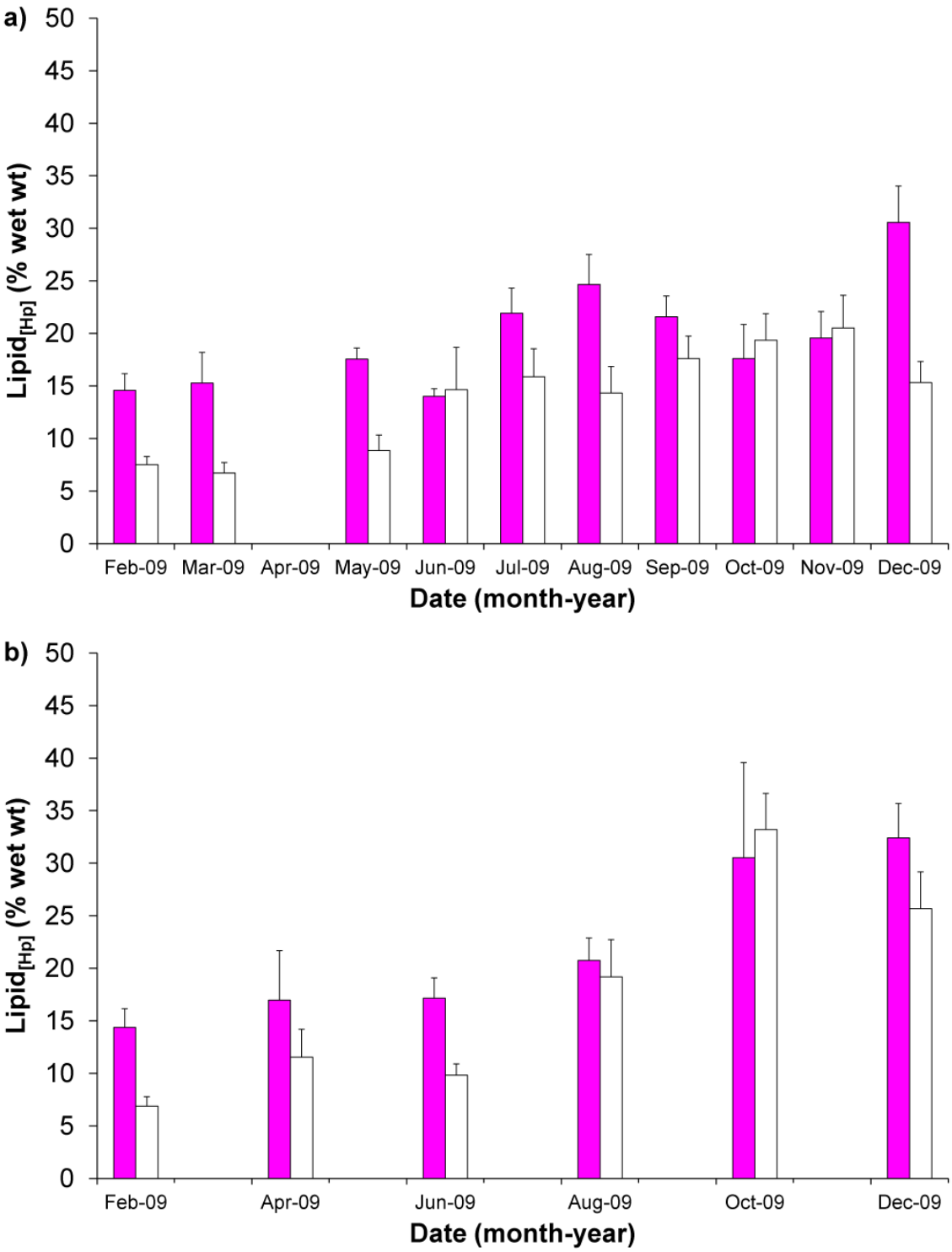

Figure 5.3 Lipid content of the hepatopancreas expressed as wet weight (means ± SE) in female (pink bars) and male (white bars) groups of *N. norvegicus* caught on the a) Clyde Sea Area- North Transect and b) North Minch- East transect throughout 2009. a) n=125, b) n=64

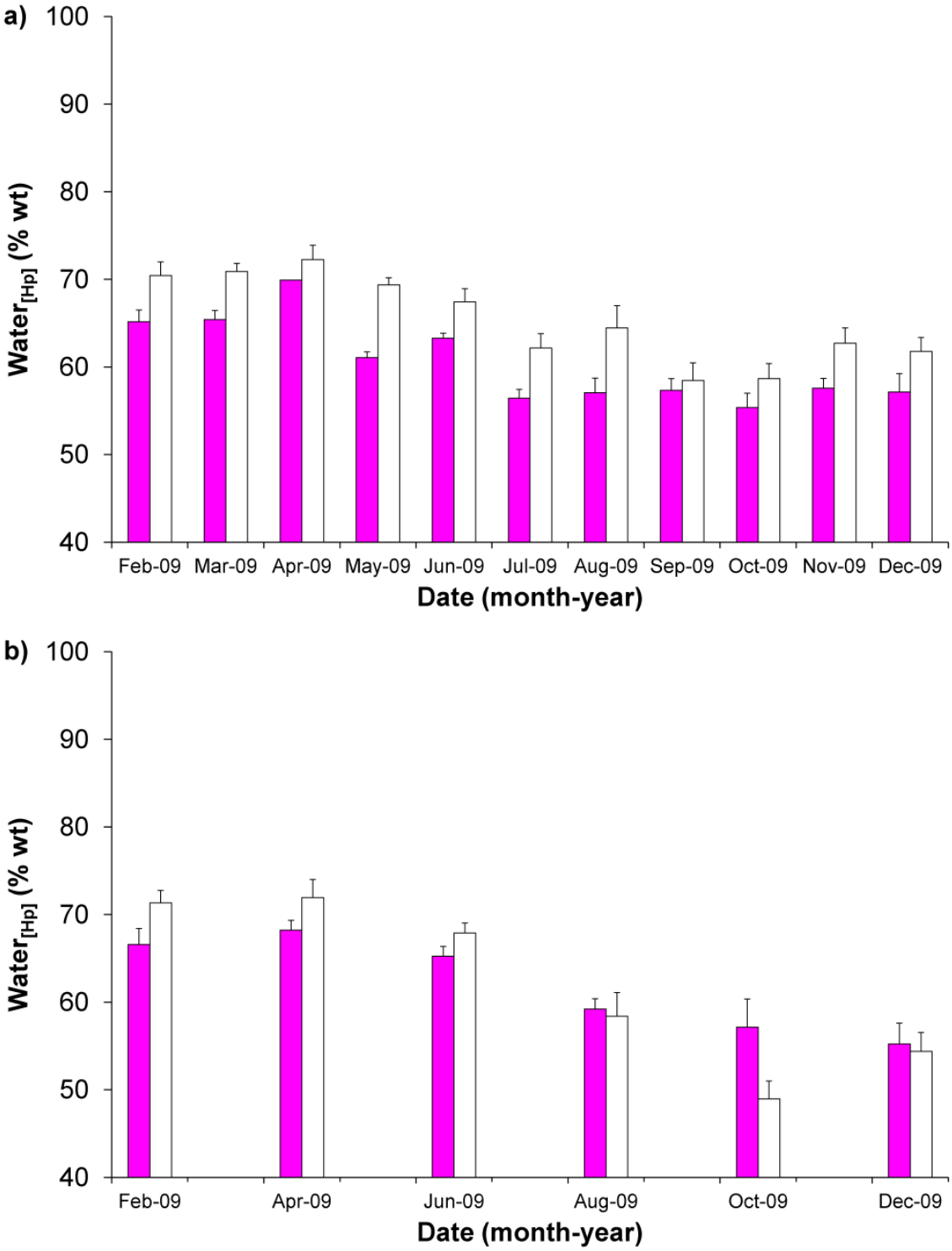

Figure 5.4 Water content of the hepatopancreas (means ± SE) in female (pink bars) and male (white bars) groups of *N. norvegicus* caught on the a) Clyde Sea Area- North Transect and b) North Minch- East transect throughout 2009. a) n=261, b) n=110

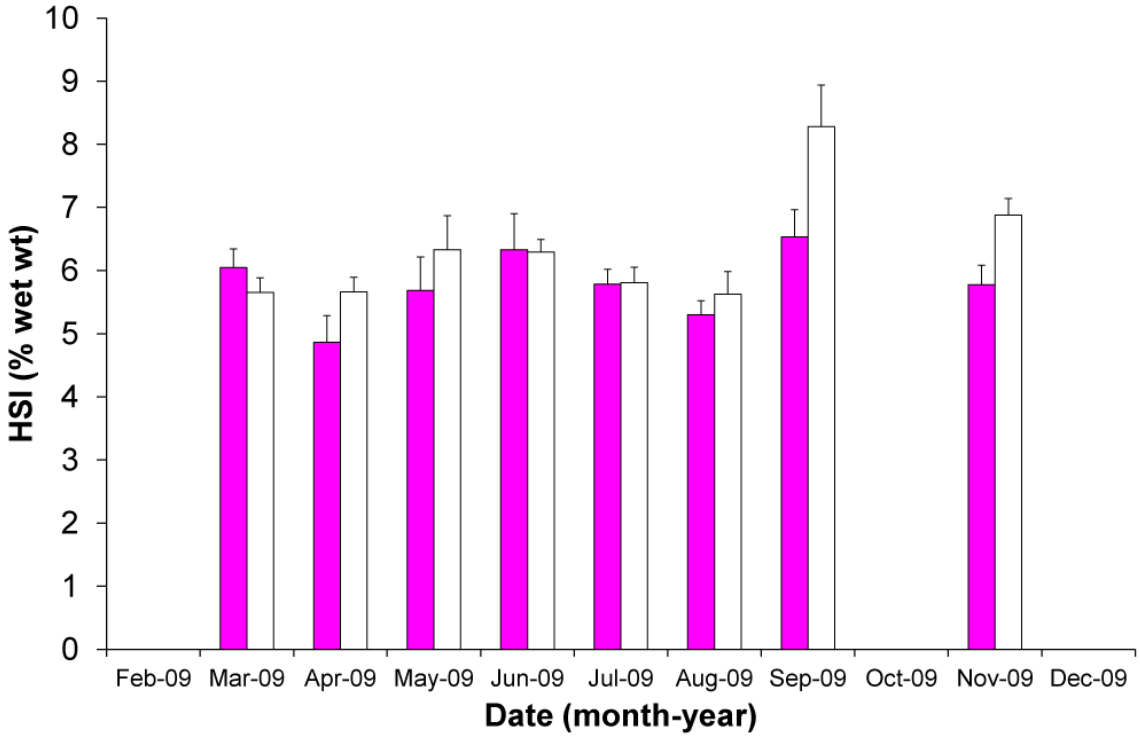

Figure 5.5 Hepatosomatic index (HSI) (means ± SE) in female (pink bars) and male (white bars) groups of *N. norvegicus* caught on the CSA (north transect) throughout 2009. n=201

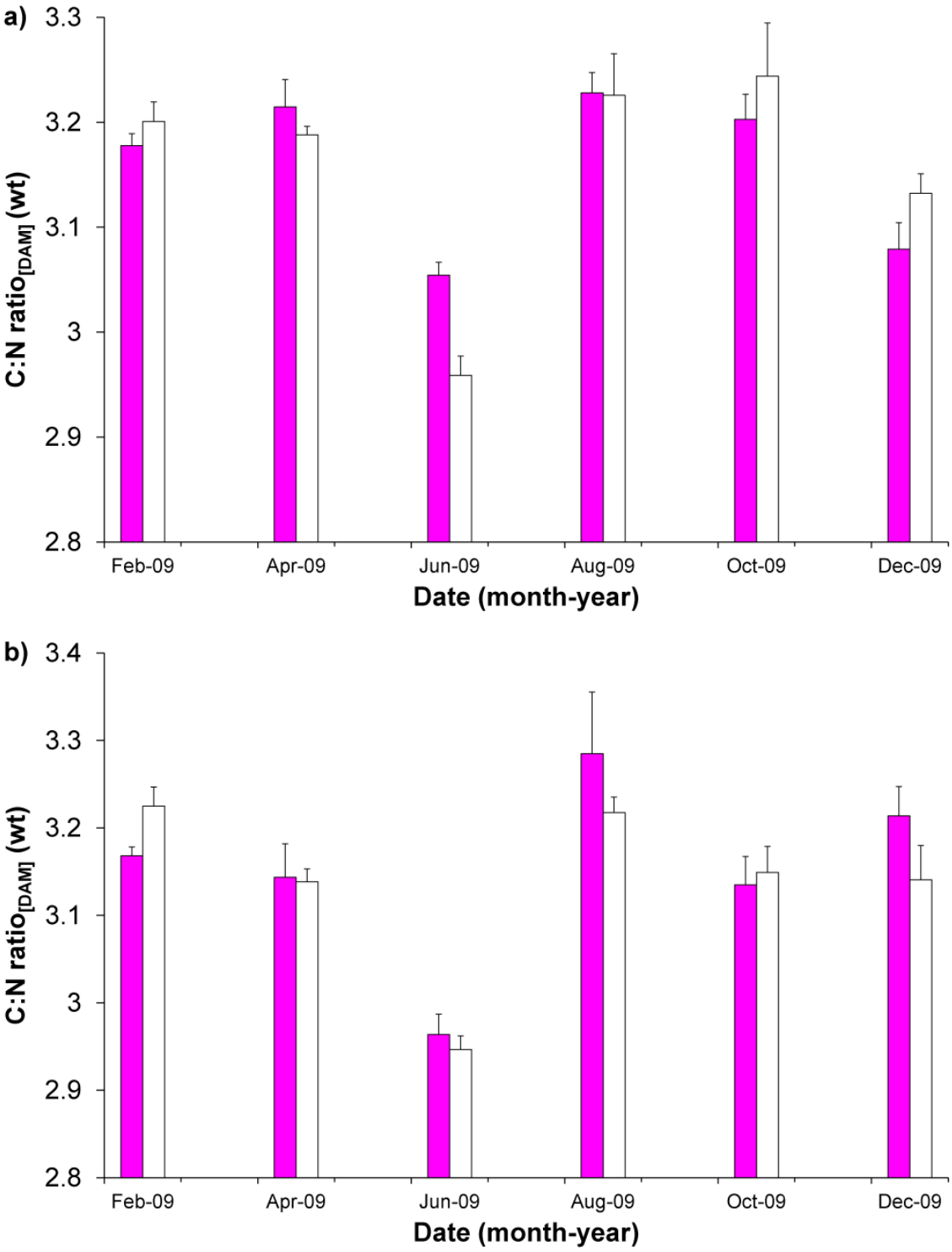

Figure 5.6 Tail muscle C:N ratio (means ± SE) in female (pink bars) and male (white bars) groups of *N. norvegicus* caught on the a) Clyde Sea Area- North Transect and b) North Minch- East transect throughout 2009. a) n=114, b) n=112

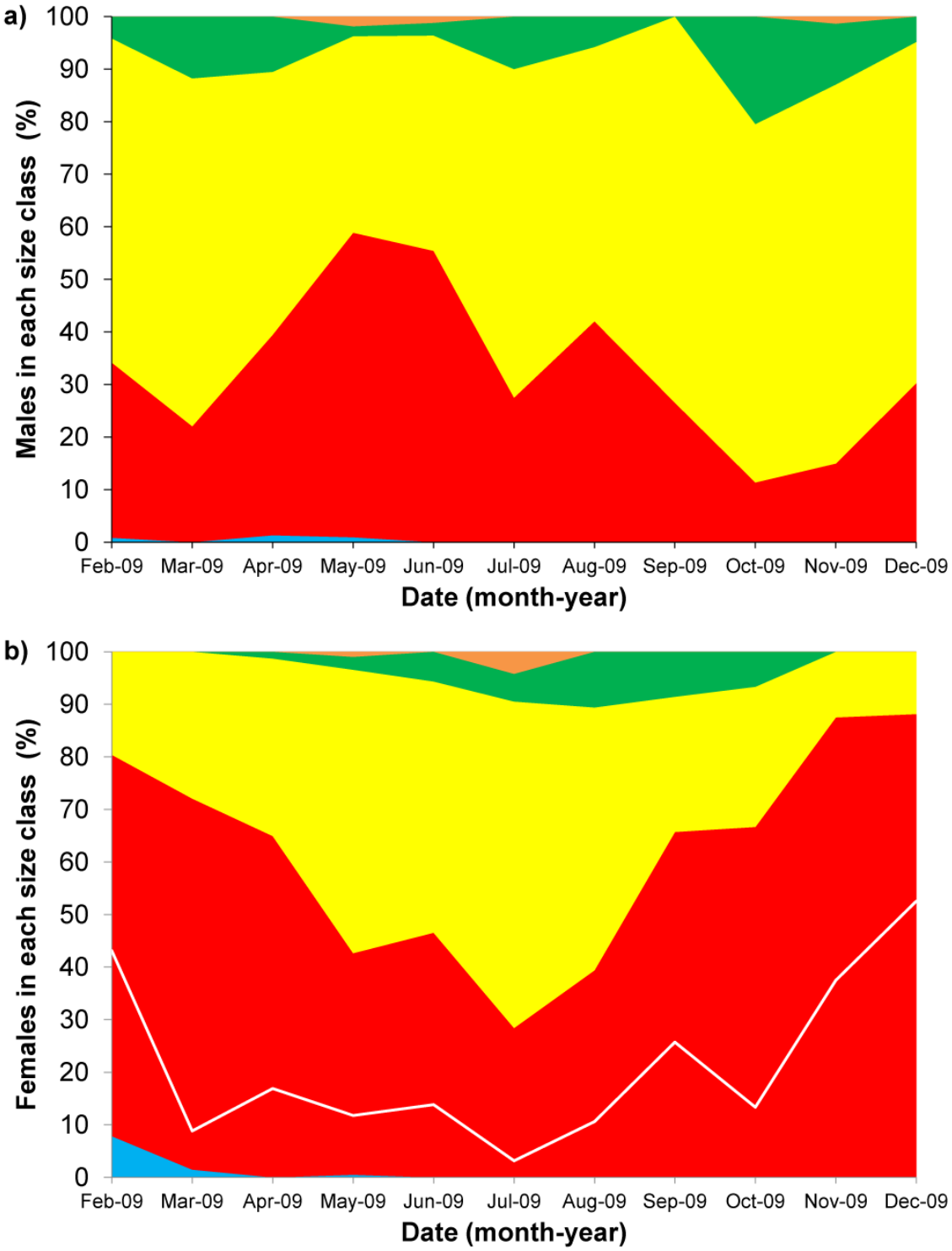

Figure 5.7 Size distribution (as carapace length) of (a) male and (b) female *N. norvegicus* from the CSA. Light blue (10-19.9mm), red (20-29.9mm), yellow (30-39.9mm), green (40-49.9mm), orange (50mm+) for a) male and b) female *N. norvegicus* from the CSA North transect. White Line represents size at onset of maturity (SOM). n=2152

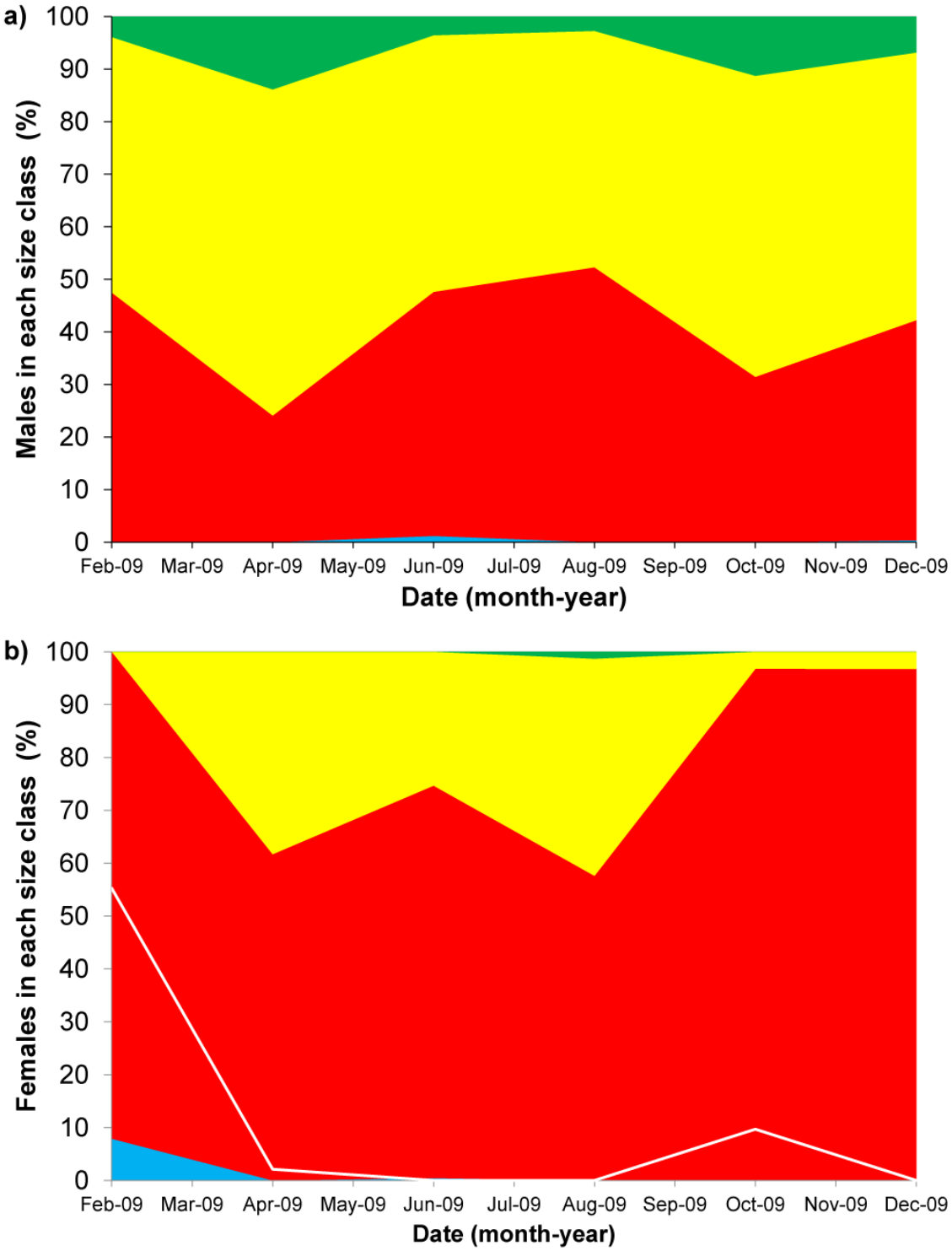

Figure 5.8 Size distribution (as carapace lengths) of (a) male and (b) female *N. norvegicus* from the NM. Light blue (10.0-19.9 mm), red (20.0-29.9 mm), yellow (30.0-39.9 mm), green (40.0-49.9 mm), orange (50 mm+). White Line represents size at onset of maturity (SOM). n=1422

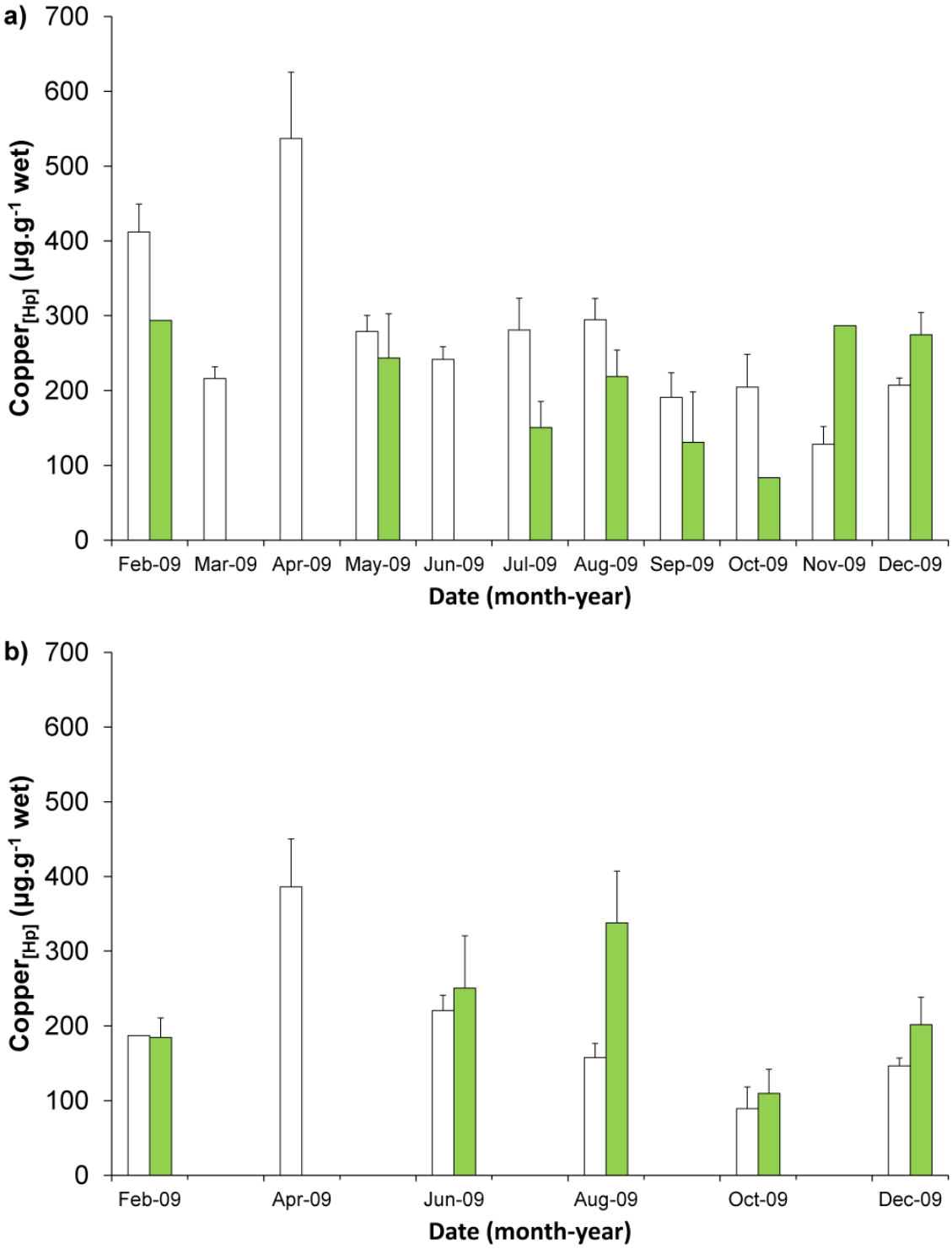

Figure 5.9 Copper content of the hepatopancreas (mean ± SE) in non ovigerous (white bars) and ovigerous (green bars) female *N. norvegicus* from a) the CSA and b) the NM throughout 2009. a) n=111, b) n=46

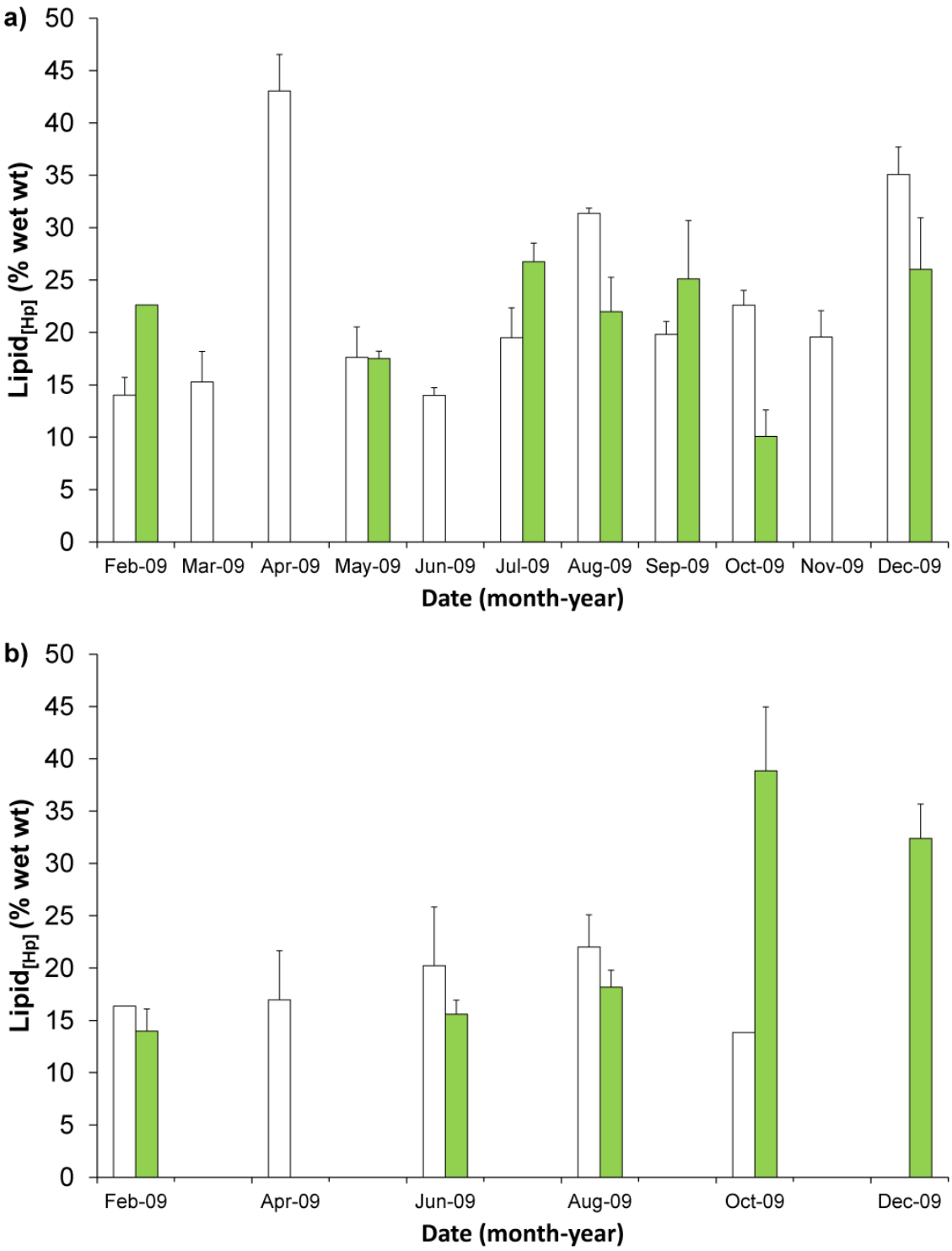

Figure 5.10 Lipid content of the hepatopancreas (mean ± SE) in non-ovigerous (white bars) and ovigerous (green bars) female *N. norvegicus* from a) the CSA and b) the NM throughout 2009. a) n=66, b) n=29

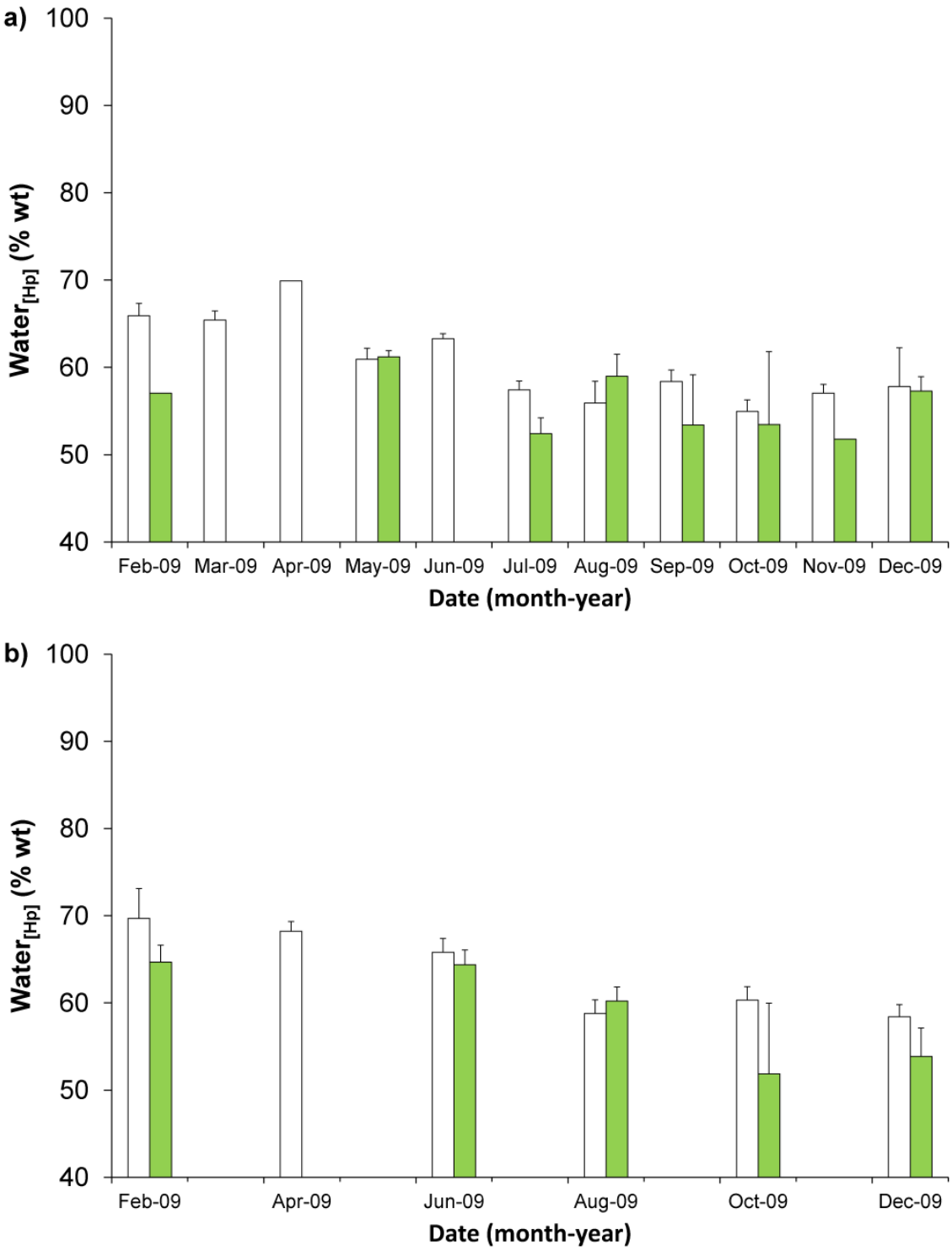

Figure 5.11 Water content of the hepatopancreas (means ± SE) in non-ovigerous (white bars) and ovigerous (green bars) female *N. norvegicus* from a) the CSA and b) the NM throughout 2009. a) n=132, b) n=51

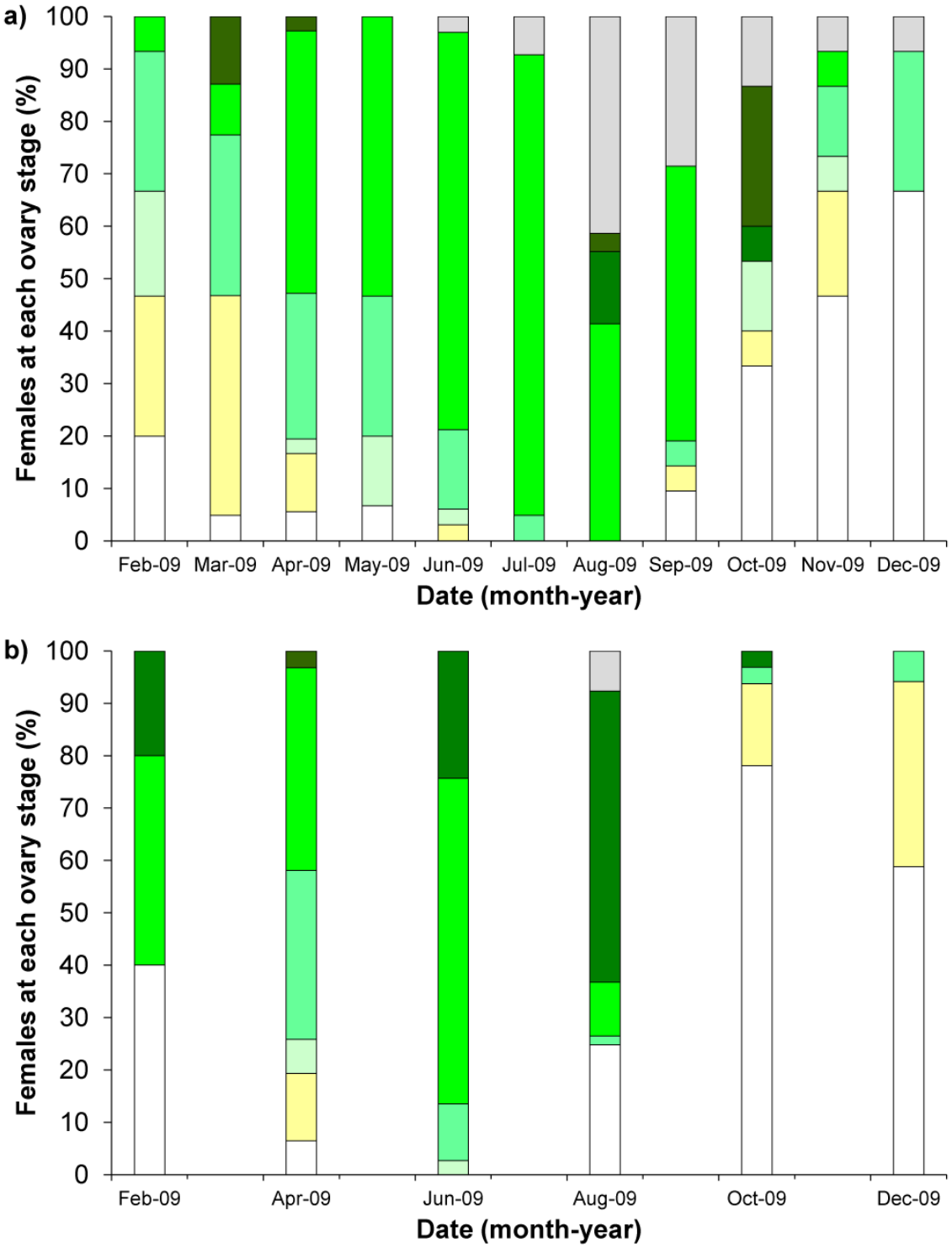

Figure 5.12 Proportions of female *N. norvegicus* caught with different staged gonads throughout the year in a) the CSA and b) the NM. White-Stage1, cream-stage 2, very pale green- stage 3, pale green- stage 4, green- stage 5, dark green 6, black (swollen with oocytes distinguishable- stage 7 and grey (reabsorbed/spent) stage 8. a) n=851, b) n=481

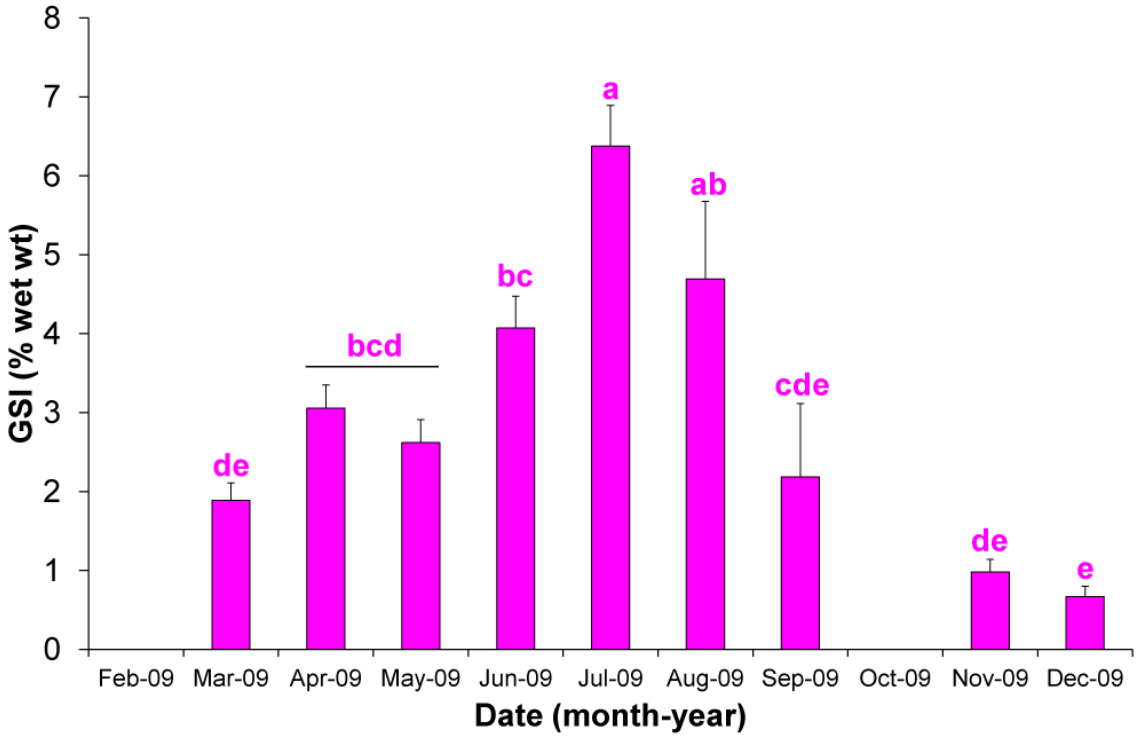

Figure 5.13 The gonadosomatic index (GSI) (mean ± SE) of female *N. norvegicus* from the CSA throughout 2009. Letters indicate the results of a post hoc Tukey test, means that do not share a letter are significantly different ($P<0.05$). n=98

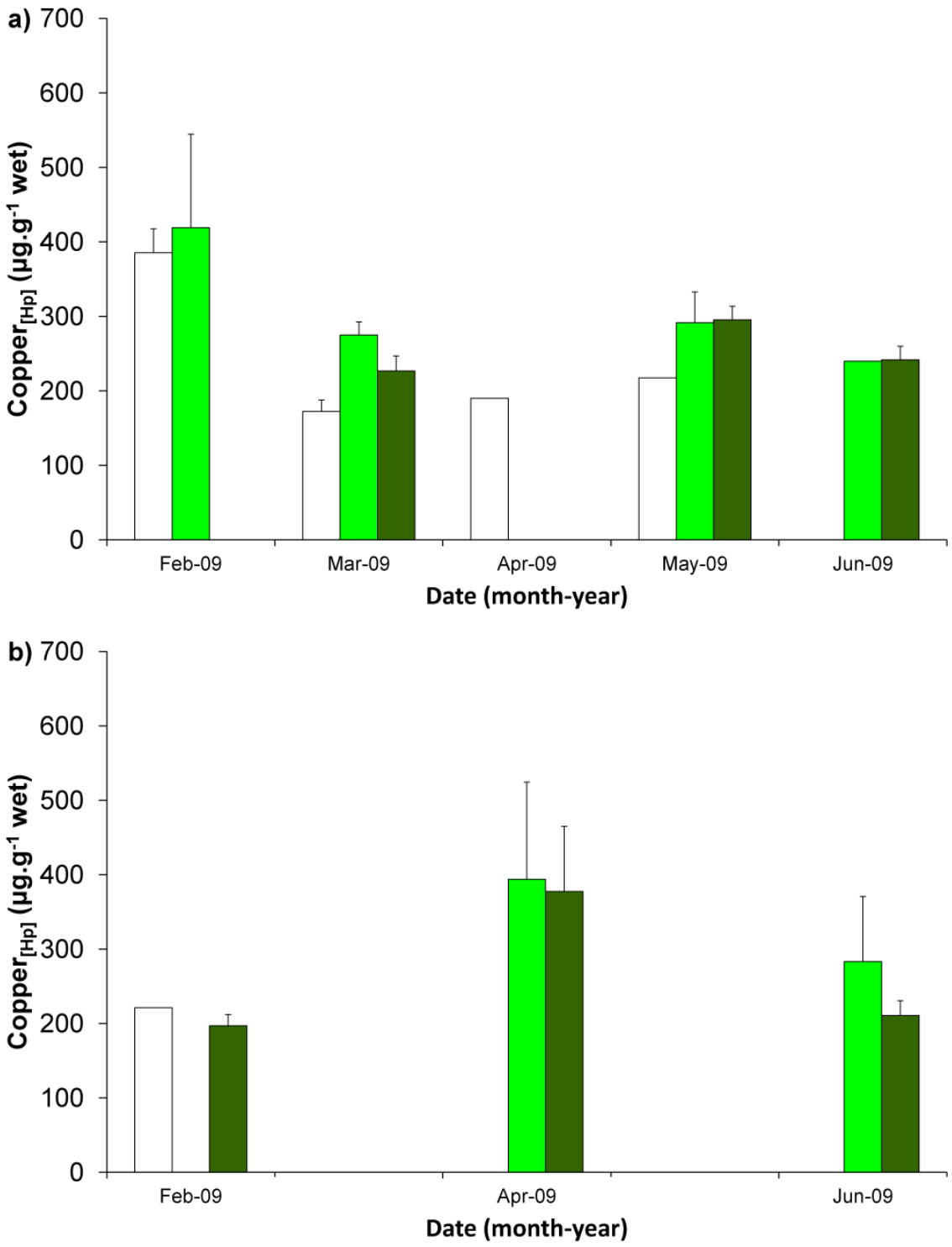

Figure 5.14 Copper concentration of the hepatopancreas (mean ± SE) female *N. norvegicus* sampled with: group A ovaries (white bars); group B ovaries (pale green bars); and group C ovaries (green bars) from a) the CSA and b) the NM between February and June 2009. a) n=44, b) n=19

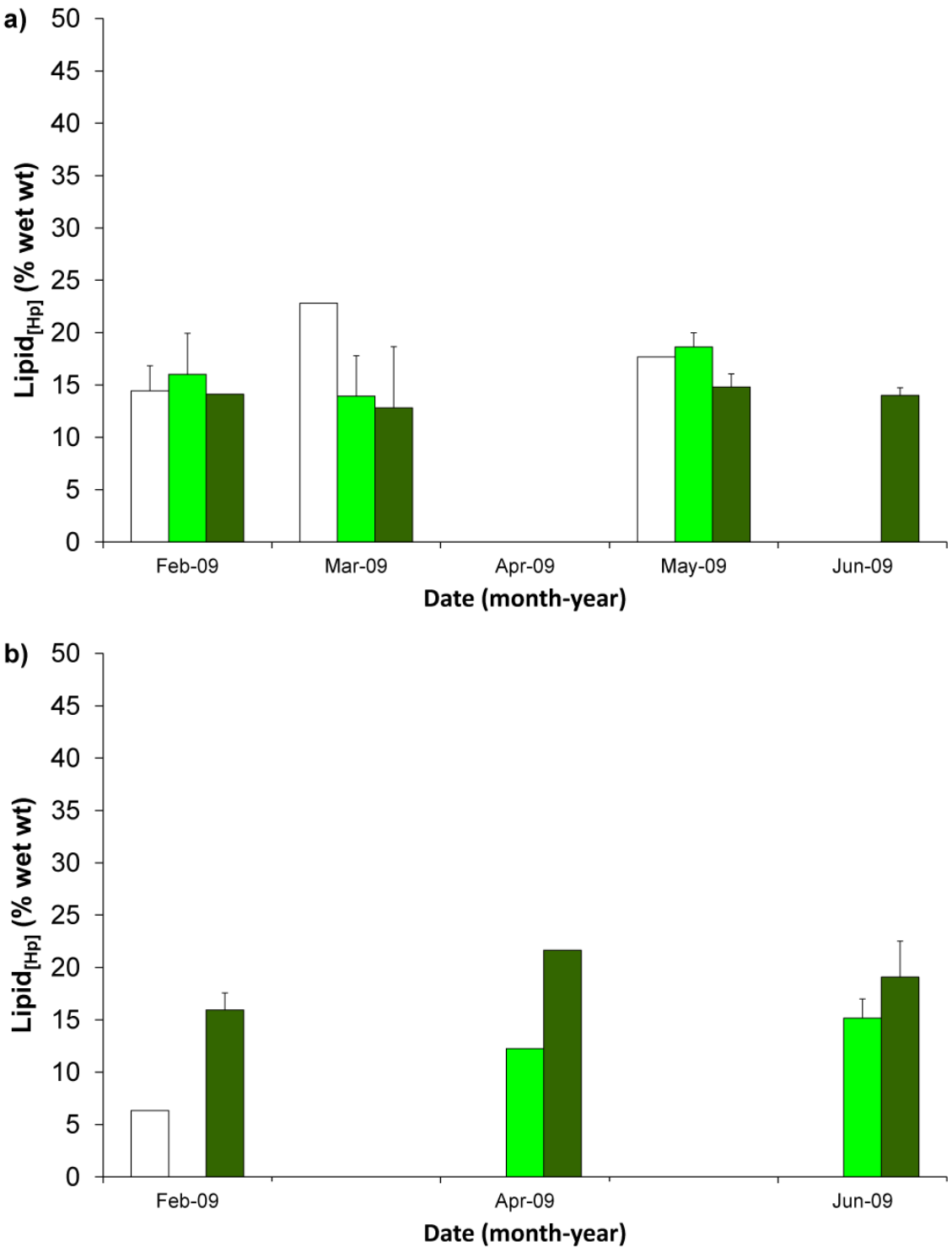

Figure 5.15 Lipid content of the hepatopancreas (mean ± SE) female *N. norvegicus* sampled with: group A ovaries (white bars); group B ovaries (pale green bars); and group C ovaries (green bars) from a) the CSA and b) the NM between February and June 2009. a) n=33, b) n=14

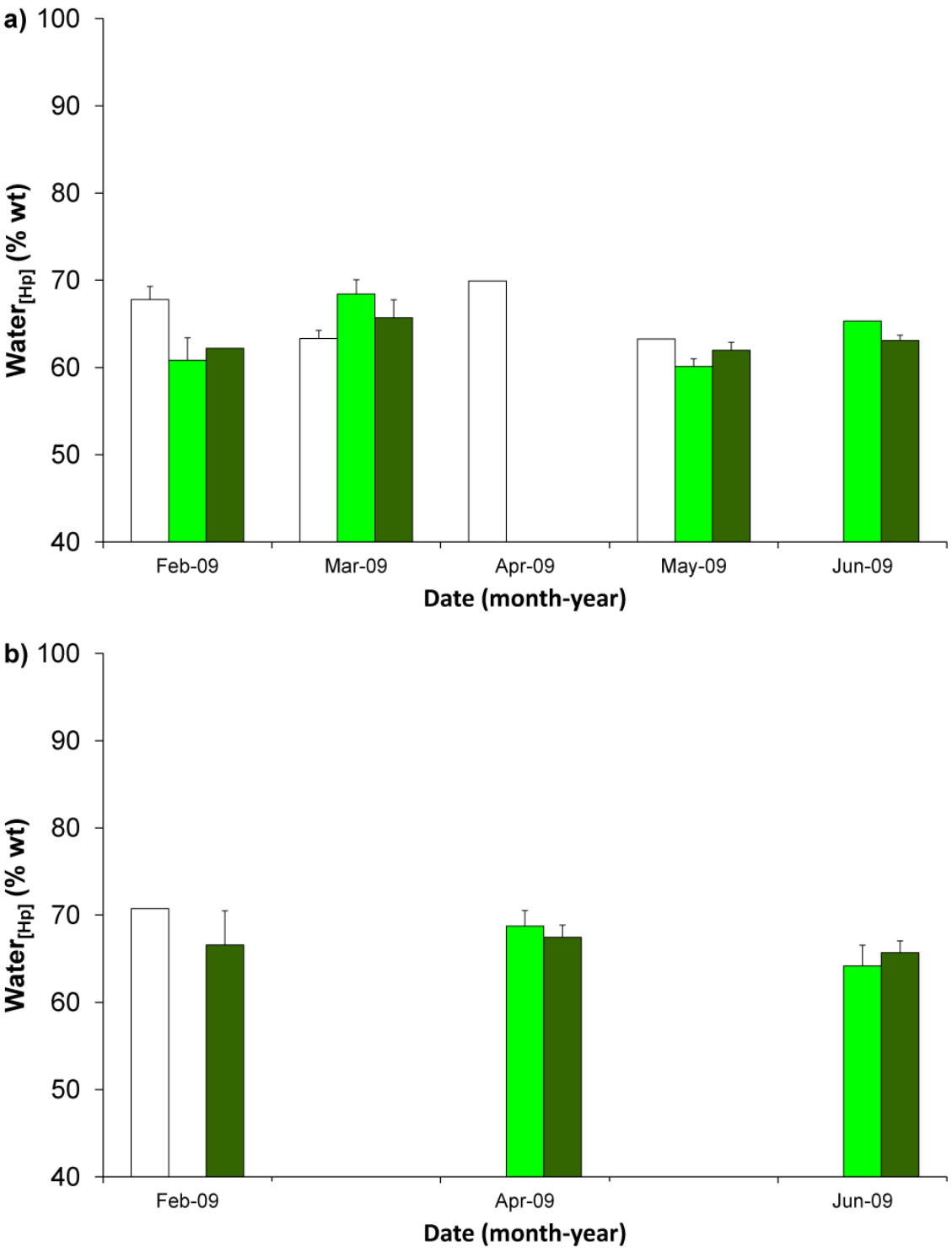

Figure 5.16 Water content of the hepatopancreas (mean ± SE) female *N. norvegicus* sampled with: group A ovaries (white bars); group B ovaries (pale green bars); and group C ovaries (green bars) from a) the CSA and b) the NM between February and June 2009. a) n=53, b) n=23

6 Trophic dynamics of *Nephrops norvegicus* off the West coast of Scotland (I): Stable isotope analysis

6.1 Introduction

To date the only thing that is known with certainty about feeding in *Nephrops norvegicus* is the identity of the food items that are detected by gut contents analysis (GCA). These data could have overestimated certain prey species due to a dominance of recently-consumed hard-structured organisms in the gut contents, as discussed in Chapter 1. This may bear little relation to the feeding process and what prey organisms are important in the overall diet of this species. Therefore alternative methods need to be employed in order in determine the feeding strategies of *N. norvegicus*.

6.1.1 Stable Isotope Analysis (SIA)

As mentioned in Chapter 1, the isotopes of nitrogen (^{15}N and ^{14}N) and carbon (^{13}C and ^{12}C) occur naturally in the tissues of living organisms and ratio of heavy to light isotopes change in predictable ways as they cycle through the biosphere (Peterson & Fry, 1987).

6.1.1.1 Theory

These isotope ratios have been used to infer dietary information in terms of trophic level and source of food web information. The isotope ^{15}N is preferentially bio-accumulated from the animal's diet, while ^{14}N is preferentially excreted by the animal. Therefore a proportional increase in ^{15}N could indicate that the animal is consuming prey at a higher trophic level than it was previously (Peterson & Fry, 1987). The isotope ^{13}C does not generally change between trophic levels, but is dependent on the flow of energy at the base of the food chain, according to the type or amount of photosynthesis that is occurring in the system (Montoya, 2007). This in a marine ecosystem indicates whether the animal is feeding on a benthic-derived food source with proportionally more ^{13}C

to ^{12}C than within a pelagic-phytoplankton derived food source, (Michener & Kaufman, 2006; Gomez-compos, 2011). The integration of the two isotope ratios can thus be used to construct ecosystem food webs and to elucidate their interactions.

6.1.1.2 Terminology

It is difficult, however, to obtain the ratios of these isotopes as their absolute abundances are difficult to measure and are prone to large error margins (Sharp, 2006). Therefore stable isotope analysis (SIA) was developed with Nier & Gulbransen (1939) being one of the first to quantify the carbon isotope ratio as an aid to determining the trophic dynamics in living systems. The isotope ratio of a sample was expressed relative to the isotope ratio of an international standard, initially by using the following equation:

$$\delta^{x}E(‰) = \left(\frac{(R_{sample})}{(R_{standard})} - 1\right)1000 \qquad \text{Equation 6.1}$$

Where:

δ- Indicates the difference in the amounts of heavy and light isotopes

E- The elemental isotope

x- The atomic mass of heavy isotope of E

R_{sample} - The ratio between the isotopes in the sample

$R_{standard}$ - A known ratio of an international standards[2].

1000 – Converts the ratio as parts per thousand (‰).

The ratios will be depicted in the standard terminology of $\delta^{15}N$ and $\delta^{13}C$

Carbon and nitrogen isotope ratios have been used in many trophic studies, and systematic reviews of this subject have been published. Peterson & Fry (1987) and Post (2002) reviewed the major literature on the use of stable isotopes in environmental trophic studies.

[2] These standards are Atmospheric Nitrogen for nitrogen (^{15}N:^{14}N ratio of $R_{standard}$ 0.365: 99.635) and 'Vienna Pee Dee Belemnitie' for Carbon (^{13}C:^{12}C 1.108:98.892), a standard derived from the original fossilised material once used (VPD) (Sulzman, 2007).

6.1.1.3 Trophic enrichment factor (TEF)

In isotope terms, the difference between a consumer and the food it consumes is known as the Trophic Enrichment Factor (TEF) where,

$$TEF\ (\Delta^{15}N,\Delta^{13}C) = \delta_{animal} - \delta_{food} \qquad \text{Equation 6.2}$$

This was estimated by Peterson and Fry (1987) to be around 3.4‰ for $\delta^{15}N$ and 0-1‰ for $\delta^{13}C$ (Minagawa & Wada, 1984; Le Loc'h & Hily, 2005; Michener & Kaufman, 2007; Post, 2002). However, this can vary due to the types of food and the efficiency of protein turnover (Michener & Kaufman, 2007). Post (2002) calculated the TEF from 56 different studies on a whole range of species from both marine and terrestrial systems. The results showed that the mean value for TEF ($\delta^{15}N$) was 3.4 ± 0.98‰ (±SD). As there is an error of almost 1‰ it is possible that the TEF for *N. norvegicus* could be different from 3.4‰. However, in comparing populations, as long as this figure is set as a constant, it would show relative trophic shifts between them. However, to calculate the Trophic Level (TL) from these values, a trophic baseline is also required.

6.1.1.4 Establishing a trophic baseline

A trophic baseline of each community is important for assessing spatial and temporal changes such as the impacts of anthropogenic inputs on the trophic dynamics of the area (Cabana & Rasmussen, 1996). Jennings & Warr (2003) stated that it is impossible to calculate TL without it. Different anthropogenic inputs have different effects on the value of $\delta^{15}N$. Heaton (1986) showed that artificial fertilizers are derived from atmospheric nitrogen and thus have similarly low effects on the base $\delta^{15}N$ value as atmospheric nitrogen. Excretions from agricultural animals and humans (washed into the sea from sewage outfalls) have relatively high $\delta^{15}N$ values in themselves and when converted to nitrate can enrich an area with $\delta^{15}N$ to around 10‰ higher than areas without this input (Cabana & Rasmussen, 1996).

A traditional way of establishing this baseline is to use the primary producer in the ecosystem. Within the marine ecosystem, phytoplankton accounts for around

90% of global marine primary production (Jennings *et al.*, 2008) and has been used in many studies to determine this trophic baseline (Le Loc'h & Hily, 2005; Hill, 2008; Sara *et al.*, 2009). The $\delta^{15}N$ value of phytoplankton can, however, vary between 6-10‰ throughout the year due to nutrient availability and light levels (Jennings & Warr, 2003). Hill (2008) showed that during the spring bloom of phytoplankton in 2004-05 the particulate organic matter (POM) decreased in the value of $\delta^{15}N$ from 7.4‰ pre-bloom to 2.9‰ during this bloom.

The use of the filter-feeding queen scallop, *Aequipecten opercularis*, as a baseline for marine benthic food webs was introduced by Jennings & Warr (2003) to alleviate the issues associated with determining a baseline from POM. They showed that this bivalve mollusc, which feeds on phytoplankton and associated materials that support benthic production, has a relatively long tissue fractionation time that helps to mask the effect of phytoplankton variability during the year. Barnes *et al.* (2009) also used this to assess the trophic baseline for $\delta^{13}C$. Other filter feeders have also been used, Unionid mussels (*Lampsilis* sp., *Anodonta* sp., and *Elliptio* sp.) were used in the same way by Cabana & Rasmussen (1996) in their study of a freshwater food web. The assumption in using these types of animals is that they will be feeding at a fixed TL, *ca* 2.5, and that they represent the community from which they are taken. The following equation will estimate TL for *N. norvegicus*:

$$TL_{sample} = \left(\frac{\delta^{15}N_{sample} - \delta^{15}N_{base}}{TEF}\right) + TL_{base}$$

Equation 6.3

Where:
Sample- the animal which TL is being calculated
Base- the organic matter/animal used as a trophic baseline

6.1.1.5 SIA in *N. norvegicus*

A number of published studies to date have examined the trophic positioning of *N. norvegicus*. Le Loc'h & Hily (2005) estimated that *N. norvegicus* in the Bay of Biscay occupied the 3rd TL. They also showed that carapace length was positively correlated with $\delta^{15}N$, which was also shown by Hill (2008) who found that *N.*

norvegicus in the western Irish Sea increased in $\delta^{15}N$ as they developed through the planktonic stages, through the juvenile stages to adulthood. She also studied adult *N. norvegicus* in 2004 through monthly sampling, showing that there was little seasonal variation. Sara *et al.* (2009) analysed demersal species off the SW Icelandic Sea, which included *N. norvegicus*. Fanelli *et al.* (2011) also included *N. norvegicus* in their analysis of the benthic community off the Catalan coast. The results of these studies, along with those of the present study, are summarised in Table 6.4.

6.1.2 Aims

The aim of this chapter is to assess the differences in the trophic level occupied by *N. norvegicus* due to a number of different factors:

- Sex (male and female) for each site separately
- Site (Clyde Sea Area-CSA and North Minch-NM)
- Season (months throughout 2009)

6.1.2.1 Sex and month interaction

As mentioned in Chapter 1, a theory has been proposed that females, when brooding eggs do not emerge to feed in the same way as they do in the summer, unlike males, which do feed throughout the year. Chapter 5 of this thesis concluded that females must continue feeding to sustain internal metabolic reserves for the development of their ovaries. Parslow-Williams (1998) and Hill (2008) discussed the possibility that these females may employ a filter feeding strategy as described by Loo *et al.* (1993), which would be possible to execute from the safety of the burrow system by trapping the suspended material as it passes the burrow entrance. If filter feeding was the predominant feeding strategy of females then the trophic level at which they are feeding will be much reduced when compared to 'normal' scavenging or predatory feeding behaviour employed by males over the winter. Therefore, in this chapter a comparison between the sexes will be made in the early summer months (as females are emerging from the burrows post-brooding) to determine if the $\delta^{15}N$ in female *N. norvegicus* is lower than the $\delta^{15}N$ of males.

6.1.2.2 Site and month interaction

In the previous studies of the isotopic ratio of $\delta^{15}N$ and $\delta^{13}C$ in *N. norvegicus*, variation among sites during the same period was not investigated. Furthermore, the way in which site differences interact with variation of the season and the sex was not addressed. A systematic comparison of these factors was therefore undertaken in the present study for the purpose of determining whether *N. norvegicus* occupies different trophic positions in different locations. This information will in turn inform the fatty acid analysis of animals from the two populations, which is the subject of the following chapter (Chapter 7).

6.2 Materials and methodology

6.2.1 Sample collection

6.2.1.1 Nephrops norvegicus

N. norvegicus collected for this SIA study were also used in the analysis of nutritional status, described in Chapter 5. The method of collection has been described in Section 5.2.1. Animals from the CSA were used only in the months in which animals were sampled from the NM. Therefore, both sites were analysed with bi-monthly sampling points to provide a direct comparison between sites. A total of ten males and ten females were selected from each bi-monthly sample.

6.2.1.2 Particulate organic matter (POM) collection

Water samples were taken in June and October 2009 via 2x 5 L Niskin water samplers triggered at either 20 m below the surface or 2 m above the bottom. The water was then filtered through a 250 µm sieve (to remove detritus, debris, sand and large zooplankton), into a plastic 5 L aspirator bottle covered in thick silver adhesive tape (to darken the inside of the bottle). Each 5 L bottle provided two 2 L sub-samples, which were then filtered through pre-combusted Whatman GF/F filter paper under a moderate vacuum. The filter paper was then held at -20˚C until required for processing.

6.2.1.3 Multi species collection

In order to replicate the trophic baseline protocol of Jennings & Warr (2003) and to test if the CSA and the NM had the same trophic base, individuals of five other species which appeared in the trawls along with *N. norvegicus* were collected from both sites within a fortnight (01 June 2010 in the CSA and 8th–11th June 2010 in the NM). The species collected were: whiting (*Merlangius merlangus*) (155-225 mm length), a demersal round fish that is known to consume crabs, shrimps, small fish, molluscs, worms, squid and cuttlefish; long rough dab (*Hippoglossoides platessoides*) (141-205 mm length) a benthic feeding flat fish which is known to feed on invertebrates and small fish; lesser spotted dogfish (*Scyliorhinus canicula*) (139-220 mm length) a demersal elasmobranch which feeds on molluscs (including small cephalopods), crustaceans, polychate worms and small teleost fish; squat lobster (*Munida rugosa*) (21.1–35.7 mm CL), a benthic decapod crustacean which is known to be an omnivore, predator and scavenger; and queen scallop (*Aequipecten opercularis*) (52–66.3 mm shell height), a filter-feeding bivalve mollusc.

6.2.2 Preparation of samples

Stable Isotope Analyses were carried out on untreated freeze-dried muscle tissues from all species and on particulate organic matter, as detailed in Chapter 2 and by Watts *et al.* (2011).

6.2.2.1 *Nephrops norvegicus* and multi species muscle tissue preparation

The tail muscle of *N. norvegicus* and *M. rugosa*, an anterior portion of the trunk muscle of *M. merlangus*, *H. platessoides*, and *S. canicula* and the adductor muscle of *A. opercularis* were freeze dried for 7 d. All hard calcareous structures were removed, along with the skin of the fish. Samples were then ground and stored in air-tight containers at -20˚C for later analysis.

6.2.2.2 Particulate Organic Matter (POM)

The filter papers with POM, prepared as in Section 6.2.1.2, were cut in half. One half was acid washed firstly in 1 ml 0.1M HCl and then 1 ml distilled water to remove any carbonates from the sample. The other half was washed in 2 ml distilled water. Each half was then dried at 60˚C for 2 hours. Samples were then scraped off the filter paper with a clean scalpel, packed inside a large tin capsule and then surrounded with another to aid combustion.

6.2.3 Measurements

Aliquots of 0.5–0.7 mg of the muscle samples were weighed into tin capsules to an accuracy of four decimal places on a Mettler MX5 balance (Mettler-Toledo international Inc., Columbus, USA). Isotope analyses were conducted at the East Kilbride node of the NERC Life Sciences Mass Spectrometry Facility hosted by Scottish Universities Environmental Research Centre (SUERC) using a continuous flow isotope ratio mass spectrometer (Thermo Fisher Delta XP Plus, Thermo Scientific Bremen, Germany) and a Costech ECS 4010 elemental analyser (Costech Analytical Technologies inc., Milan, Italy). Drift and linearity were corrected by the use of laboratory standards of gelatine (reproducibility (s.d.) of around 0.18‰ for $\delta^{15}N$ and 0.10‰ for $\delta^{13}C$) and alanine, which are routinely checked against international isotope standards from the International Atomic Energy Agency (IAEA) and National Institute of Standards and Technology (NIST). $\delta^{15}N$ and $\delta^{13}C$ was calculated in the standard way according to Equation 6.1. The two splits of particulate organic matter were run in tandem, with the acid-washed sample being used for determining $\delta^{13}C$ and the non-acid-washed sample being used for determining $\delta^{15}N$.

6.2.4 Statistical analysis

The data were analysed with a general linear model, with subsequent post hoc Tukey test for significant interactions. Normality of residuals was assessed visually. Errors displayed are standard errors unless otherwise stated.

Differences between two $\Delta^{x}E$ of the same isotope between two groups of animals will be noted in the standard way:

$\Delta^{x}E_{group1\text{-}group2}$ Equation 6.4

Where:
$\Delta^{x}E$ is the isotope in question, i.e. $\delta^{15}N$ or $\delta^{13}C$
And group1-2 gives the different levels for each factor, e.g. $\delta^{15}N_{male\text{-}female}$

6.3 Results

6.3.1 Site differences

The values of $\delta^{15}N$ and $\delta^{13}C$ obtained from the tail muscle of male *N. norvegicus* selected in February 2009 from all four transects (two in the CSA and two in the NM) are shown in Figure 6.1.

Comparing sites around one date in February 2009, there was a significant difference between the mean values of $\delta^{15}N$ in the tail muscles of animals from the CSA (12.40 ± 0.09‰) and from the NM (10.75 ± 0.06‰) ($F_{1,37}$=208.60, P<0.001). There was also a significant difference between the mean values of $\delta^{13}C$ in the tail muscles of animals from the CSA (-15.63 ± 0.05‰) and from the NM (-16.43 ± 0.04‰) ($F_{1,37}$=154.29, P<0.001).

Comparing transects, in the CSA there was a significant difference between the mean values of $\delta^{15}N$ in the tail muscles of animals from the north transect (12.61 ± 0.07‰) and from the west transect (12.18 ± 0.015‰) ($F_{1,19}$=6.74, P=0.018). There was also a small but significant difference between the mean values of $\delta^{13}C$ in the tail muscles of animals from the north transect (-15.52 ± 0.06‰) and from the west transect (-15.72 ± 0.06‰) ($F_{1,19}$=6.33, P=0.022). In the NM there was no significant difference between the mean values of $\delta^{15}N$ in the tail muscles of animals from the east transect (10.85 ± 0.08‰) and from the south transect (10.68 ± 0.08‰) ($F_{1,17}$=4.13, P=0.059). There was also no significant difference between the mean values of $\delta^{13}C$ in the tail muscles of animals from

the east transect (-16.42 ± 0.06‰) and from the south transect (-16.44 ± 0.07‰) ($F_{1,17}$=0.05, P=0.822).

As in the last chapter, due to limitations on the number of samples that could be processed, one transect was chosen to represent each site. The choice of transect was first determined by the smallest variation within the data (represented by standard error, SE). Using this criterion, the north transect was chosen to represent the CSA (and will hereafter in this chapter be referred to as the CSA), and the east transect was chosen to represent the NM (and will hereafter in this chapter be referred to as the NM).

6.3.2 Sex - month interaction

To compare the effect of sex, the two sites were analysed separately to remove the influence of site. Means (± SE) of all terms are shown in Table 6.1.

6.3.2.1 Clyde Sea Area

The mean values (± SE) of δ^{15}N and δ^{13}C in the tail muscles of male and female *N. norvegicus* selected bi-monthly throughout 2009 from the CSA are shown in Figure 6.2.

There was a significant effect of the interaction between sex and month of δ^{15}N in the tail muscles of *N. norvegicus* caught in the CSA ($F_{5,118}$=4.75, P=0.001). The post hoc Tukey test indicated that females in December (13.38 ± 0.06‰) have a statistically more positive δ^{15}N than males (12.72 ± 0.11‰). For the rest of the year there was no significant difference between males and females at each bimonthly sampling point.

There was no significant effect of the interaction between sex and month of δ^{13}C in the tail muscles of *N. norvegicus* caught in the CSA ($F_{5,118}$=0.82, P=0.536). There was no significant difference in the annual average δ^{13}C between males and females ($F_{1,118}$=2.41, P=0.124). There was however a significant difference in the δ^{13}C amongst the bi-monthly samples ($F_{5,118}$=2.85, P=0.019). The post hoc Tukey test indicated that *N. norvegicus* caught in October (-15.68 ± 0.06‰) and

February (-15.70 ± 0.03‰) had statistically more negative $\delta^{13}C$ than *N. norvegicus* in June (-15.41 ± 0.04‰) and April (-15.41 ± 0.5‰) (Tukey, P<0.05).

6.3.2.2 North Minch

The mean values (± SE) of $\delta^{15}N$ and $\delta^{13}C$ in the tail muscles of male and female *N. norvegicus* selected on a bi-monthly basis throughout 2009 from the NM are shown in Figure 6.3.

There was a significant effect of the interaction between sex and month of $\delta^{15}N$ in the tail muscles of *N. norvegicus* caught in the NM ($F_{5,119}$=2.60, P=0.029). The post hoc Tukey test indicated that females (10.81 ± 0.23 ‰) have a significantly less positive $\delta^{15}N$ than males (11.72 ± 0.26‰) in December 2009, which is the opposite of what was found in the CSA samples.

There was no significant effect of the interaction between sex and month of $\delta^{13}C$ in the tail muscles of *N. norvegicus* caught in the NM ($F_{5,119}$=1.25, P=0.290). The months August, October and December are more negative than the months February, April, and June in both males and females, when comparing both sexes together ($F_{5,119}$=23.00,P<0.001) (Tukey P<0.05).

Table 6.1 Means (± SE) of a) $\delta^{15}N$ (‰) and b) $\delta^{13}C$ (‰) in female and male *N. norvegicus* throughout the year from the CSA and the NM. $\Delta^{x}E_{female\text{-}male}$ indicates the difference between females and males. Month groups per measure that do not share a letter are significantly different. Letters within different measures are different and cannot be cross-referred.

		CSA			NM		
		Females	**Males**	**$\Delta^{x}E_{female\text{-}male}$**	**Females**	**Males**	**$\Delta^{x}E_{female\text{-}male}$**
$\delta^{15}N$ (‰)	**Feb-09**	12.89 ± 0.09 **abc**	12.71 ± 0.07 **bc**	0.18	10.84 ± 0.10 **b**	11.05 ± 0.20 **ab**	-0.22
	Apr-09	13.03 ± 0.14 **ab**	12.77 ± 0.09 **bc**	0.26	10.82 ± 0.09 **b**	11.04 ± 0.10 **ab**	-0.22
	Jun-09	13.30 ± 0.09 **a**	12.49 ± 0.21 **c**	0.81	10.79 ± 0.27 **b**	10.49 ± 0.16 **b**	0.30
	Aug-09	13.11 ± 0.09 **ab**	13.14 ± 0.07 **ab**	-0.03	11.20 ± 0.09 **ab**	11.21 ± 0.10 **ab**	-0.01
	Oct-09	13.15 ± 0.10 **ab**	13.00 ± 0.08 **ab**	0.15	11.14 ± 0.19 **ab**	11.16 ± 0.19 **ab**	-0.02
	Dec-09	13.38 ± 0.06 **a**	12.72 ± 0.11 **bc**	0.66	10.81 ± 0.23 **b**	11.72 ± 0.26 **a**	-0.91
	Annual	**13.14 ± 0.09**	**12.80 ± 0.10**	**0.34**	**10.92 ± 0.16**	**11.05 ± 0.06**	**-0.13**
$\delta^{13}C$ (‰)	**Feb-09**	-15.66 ± 0.07	-15.74 ± 0.07	0.19	-16.11 ± 0.06	-16.33 ± 0.11	0.22
	Apr-09	-15.56 ± 0.14	-15.28 ± 0.03	-0.28	-15.93 ± 0.08	-16.20 ± 0.07	0.27
	Jun-09	-15.46 ± 0.07	-15.36 ± 0.09	-0.1	-16.31 ± 0.16	-16.14 ± 0.05	-0.17
	Aug-09	-15.59 ± 0.17	-15.56 ± 0.15	-0.03	-16.66 ± 0.11	-16.68 ± 0.09	0.02
	Oct-09	-15.69 ± 0.09	-15.67 ± 0.13	-0.02	-16.84 ± 0.07 **c**	-16.88 ± 0.07 **c**	0.04
	Dec-09	-15.62 ± 0.09	-15.41 ± 0.06	-0.21	-16.74 ± 0.12 **c**	-16.82 ± 0.14 **c**	0.08
	Annual	**-15.60 ± 0.11**	**-15.51 ± 0.03**	**-0.09**	**-16.43 ± 0.05**	**-16.51 ± 0.09**	**0.08**

6.3.3 Site - month interaction

As there was little variation between the sexes when comparing sites separately to compare the difference between sites, the sexes were combined.

The mean values (± SE) of $\delta^{15}N$ and $\delta^{13}C$ in the tail muscles of *N. norvegicus* selected on a bi-monthly basis throughout 2009 between the CSA and the NM are shown in Figure 6.4.

There was no significant effect of the interaction between site and month of the $\delta^{15}N$ of the tail muscles of *N. norvegicus* ($F_{5,238}$= 1.05, P=0.390). However there was a significant difference in the $\delta^{15}N$ values between sites ($F_{1,238}$=907.60, P<0.001), with the animals from the CSA having an annual average $\delta^{15}N$ of 12.97 ± 0.03‰ and the animals from the NM having an annual average of 11.02 ± 0.05‰.

There was a significant effect of the interaction between site and month of the $\delta^{13}C$ of the tail muscles of *N. norvegicus* ($F_{5,238}$=8.83, P<0.001). The post hoc Tukey test indicated in each month *N. norvegicus* sampled from the NM had significantly more negative $\delta^{13}C$ than *N. norvegicus* from the CSA there was a significant difference.

6.3.4 Site difference of multiple species

The mean values (± SE) of $\delta^{15}N$ and $\delta^{13}C$ in all the species selected for the multi-species comparison on 01 June 2010 in the CSA and 8-11 June 2010 in the NM are shown in Figure 6.5.

There was a significant effect of the interaction between species and site of the $\delta^{15}N$ of the animals tissues ($F_{5,67}$=8.10, P<0.001). The post hoc Tukey test results (shown in Table 6.2) indicate that, the individuals of each species selected from the CSA all had significantly higher $\delta^{15}N$ values than the individuals of the same species selected from the NM. There was no significant difference in $\delta^{15}N$ between *M. rugosa* and *N. norvegicus* from the NM, although there was a significant difference between these two species sampled from the CSA.

There was no significant effect of the interaction between species and site of the $\delta^{13}C$ of the animals tissues ($F_{5,67}$=1.69, P=0.152). However there was a significant difference in the $\delta^{13}C$ values among all species ($F_{5,67}$=44.84, P<0.001) and between sites ($F_{1,67}$=183.99, P<0.001)

Table 6.2 The results of the post hoc Tukey test of multiple species measured in the Clyde Sea Area (CSA) and in the North Minch (NM) of the interaction between site and species. Species that do not share a letter between sites are significantly different. n=68

	Species	**CSA**	**NM**
$\delta^{15}N$ (‰)	*S. canicula*	16.83 **ab**	12.40 ± 0.13 **ef**
	M. merlangus	16.43 ± 0.17 **a**	13.90 ± 0.16 **de**
	H. platessoides	15.34 ± 0.21 **bc**	12.40 ± 0.13 **f**
	M. rugosa	14.40 ± 0.16 **cd**	11.27 ± 0.15 **g**
	A. opercularis	10.92 ± 0.13 **g**	7.19 ± 0.41 **h**
	N. norvegicus	12.89 ± 0.20 **f**	11.18 ± 0.15 **g**

6.3.4.1 Variation in the difference in species between sites

The differences between the means of each species from the CSA and the NM are shown in Figure 6.6. This shows that there is a relatively small difference seen in *N. norvegicus* between the CSA and the NM compared with the other species selected. The site difference between the means of each species were whiting, *M. merlangus* ($\Delta^{15}N_{CSA-NM}$ 2.53‰, $\Delta^{13}C_{CSA-NM}$ 0.91‰), long rough dab, *H. platessoides* ($\Delta^{15}N_{CSA-NM}$ 2.94‰, $\Delta^{13}C_{CSA-NM}$ 1.39‰); lesser spotted dogfish, *S. canicula* ($\Delta^{15}N_{CSA-NM}$ 3.91‰, $\Delta^{13}C_{CSA-NM}$ 1.84‰); squat lobster, *M. rugosa* ($\Delta^{15}N_{CSA-NM}$ 3.13‰, $\Delta^{13}C_{CSA-NM}$ 0.99‰); Norway lobster, *N. norvegicus* ($\Delta^{15}N_{CSA-NM}$ 1.71‰, $\Delta^{13}C_{CSA-NM}$ 1.32‰); queen scallop, *A. opercularis* ($\Delta^{15}N_{CSA-NM}$ 3.74‰, $\Delta^{13}C_{CSA-NM}$ 1.43‰).

6.3.5 Determination of trophic level

6.3.5.1 Particulate organic matter (POM)

The values of $\delta^{15}N$ and $\delta^{13}C$ in the particulate organic matter (POM) sampled in June and October 2009 from the CSA and the NM are shown in Figure 6.7.

There was no significant difference in the POM values between sites ($F_{1,11}$=0.13, P=0.724). However, there was too much variation in the POM data to establish effectively the trophic baseline for each site, thus it was impossible to determine trophic position of *N. norvegicus* between sites using POM.

6.3.5.2 Aequipecten opercularis

There was a significant difference between the mean $\delta^{15}N$ values of *A. opercularis* between the CSA (10.92 ± 0.13‰) and the NM (7.19 ± 0.41‰) ($F_{1,8}$=132.40, P<0.001). Jennings & Warr (2003) noted that *A. opercularis* has a TL of 2.5.

When these values are combined with the values for *N. norvegicus* selected at the same time and placed into the trophic level equation (Equation 6.3). There was no significant difference between the $\delta^{15}N$ values of *N. norvegicus* sampled at the two sites either in the various months of 2009 or in June 2010 (Tukey >0.05). The values of *A. opercularis* sampled in June 2010 could therefore (tentatively) be used as a base to calculate an 'Approximate Trophic Level' (ATL) for all other males throughout 2009. This is approximate as the values of *A. opercularis* in the CSA and the NM are not known for 2009 and how much they would vary from the $\delta^{15}N$ value of June 2010, although it could be expected to be round about ± 1.5‰ of the value calculated (Jennings & Warr (2003).

The mean values of the ATL for male *N. norvegicus* selected from the CSA and the NM in 2009 and June 2010 are shown in Figure 6.8. There was a significant difference in the ATL in male *N. norvegicus* with site ($F_{1,225}$= 990.15, P<0.001), with the animals from the CSA having an average ATL of 3.1 and the animals from the NM having an average of 3.6. There was also a significant difference in the ATL of *N. norvegicus* amongst the bi-monthly samples ($F_{6,225}$=9.93, P<0.001).

There was a significant difference in the interaction of month and site ($F_{6,225}$=3.10, P=0.006). This shows that there is a clear difference between the animals from the CSA and the NM.

Between-month comparisons of the NM *N. norvegicus* show that in December they were feeding at a significantly higher TL than *N. norvegicus* in February and April (Tukey, P<0.05), whereas *N. norvegicus* in June were at a lower TL than in all other months. Between-month comparisons in the CSA animals show that animals sampled in August and October 2009 had a significantly higher TL than in other months; again the *N. norvegicus* sampled in June in the CSA had a lower TL than *N. norvegicus* sampled in August and October. Table 6.4 shows the mean TLs for the CSA and the NM, along with the values for a number of other sites across the range occupied by *N. norvegicus*.

The TL of each of the other species is shown in Figure 6.9. The lesser spotted dogfish was removed from further consideration as only one individual was obtained from the CSA. There was a significant effect of the interaction of species and site ($F_{3,53}$=6.69, P=0.001). *M. rugosa* and *N. norvegicus* in the NM were at the same TL as each other (*M. rugosa* = 3.7, *N. norvegicus* = 3.6) whereas in the CSA the TLs of these two species were significantly different (*M. rugosa* = 3.5, *N. norvegicus* = 3.1).

Table 6.3 The mean (± SE) of the Approximate Trophic Level (ATL) for *N. norvegicus* sampled from the CSA and NM throughout 2009.With a post hoc Tukey test on the interaction between month and site for animals caught in the CSA and NM. Groups that do not share a letter are significantly different.

		CSA	**NM**
ATL	Feb-09	3.03 ± 0.01 **de**	3.64 ± 0.04 **b**
	Apr-09	3.04 ± 0.02 **de**	3.63 ± 0.02 **b**
	Jun-09	2.96 ± 0.04 **e**	3.47 ± 0.03 **c**
	Aug-09	3.15 ± 0.01 **d**	3.68 ± 0.02 **ab**
	Oct-09	3.11 ± 0.02 **d**	3.67 ± 0.05 **ab**
	Dec-09	3.03 ± 0.03 **de**	3.83 ± 0.08 **a**
	Jun-10	3.08 ± 0.06 **de**	3.67 ± 0.05 **ab**

6.4 Discussion

In this chapter the values of $\delta^{15}N$ and $\delta^{13}C$ for *N. norvegicus* in the two sites studied in Chapter 5, and the two factors of sex and season were considered.

6.4.1 Sex

In the interaction between sex and month the only significant difference between males and females was that of $\delta^{15}N$ in animals in December when male *N. norvegicus* from the CSA had significantly more positive $\delta^{15}N$ than females. In the NM this was the other way round (with the males having significantly more positive $\delta^{15}N$ than females). In all other month there was no significant difference. And there was no significant difference in $\delta^{13}C$ between male and females in any month on any site.

The variation in $\delta^{13}C$ in a marine environment indicates the source of energy supporting the food web (Barnes *et al.*, 2009; Montoya, 2007). These results suggest that for animals in the same area the sources within the food webs for male and female *N. norvegicus* are the same.

Parslow-Williams (1998) suggested female *N. norvegicus* may utilise a filter feeding strategy through the brooding period, instead of emerging from the burrow to feed via a scavenging or predatory feeding strategy. If this hypothesis were true, females would be significantly lower in $\delta^{15}N$ than their male counterparts; especially around the months of April and June. The lower $\delta^{15}N$ value would then signify a lower trophic level position. This, however, was not seen in this present study, therefore there is no evidence to support the hypothesis that females are filter feeding while in their burrows.

6.4.2 Site

The $\delta^{13}C$ values obtained from *N. norvegicus* differed between the CSA and NM. The $\Delta^{13}C_{CSA\text{-}NM}$ was 0.85‰ with the CSA having a less negative $\delta^{13}C$ (-15.55‰) than the NM (-16.40‰).

In a marine system, a more negative $\delta^{13}C$ can indicate a more pelagic-based diet (Boulton, 1991; Michener & Kaufman, 2007; Barnes *et al.*, 2009). Boulton (1991) showed that phytoplankton will have a $\delta^{13}C$ of *ca* -22‰ and *ca* 15‰ in benthic macroalgae, suggesting that the animals from the NM are feeding within a more pelagic driven food web than the animals in the CSA. The abiotic factors of salinity, temperature and depth can change the rate of CO_2 fixation in marine primary producers (Boulton, 1991; Fry, 1996; Barnes *et al.*, 2009). Barnes *et al.* (2009) showed that $\delta^{13}C$ becomes more negative with depth. Boulton (1991) suggested that ^{13}C-depleted organic matter sinks to the deeper depths, resulting in a more negative $\delta^{13}C$.

In this multi-species study, all animals caught in the NM also had a more negative $\delta^{13}C$ than the individuals of the same species in the CSA. Therefore, the difference in $\delta^{13}C$ is not restricted to the particular feeding habits of *N. norvegicus* but is due to a site difference. This may, in part, be due to the

greater depth of the site sampled in the NM and that the NM food web is derived from a more pelagic source, whist in the CSA it is derived from a more benthic based diet. This, however, will be studied further in Chapter 7 where the food types consumed by these animals will be considered.

6.4.2.1 Trophic level differences between sites

The trophic level of *N. norvegicus* from two areas off the west coast of Scotland was determined with the use of $\delta^{15}N$. This demonstrated the importance of measuring the trophic baseline for food webs at different locations when utilising stable isotope ratios to determine trophic level. Post (2002) stated that one organism alone provides very little information about the absolute trophic position of the species.

The $\delta^{15}N$ values obtained from *N. norvegicus* from the CSA and NM were significantly different from each other. $\Delta^{15}N_{CSA-NM}$ was 1.91‰ with the animals selected from the CSA having an average $\delta^{15}N$ of 12.96‰ and the NM having an average $\delta^{15}N$ of 10.98‰, both with sex and month combined.

In the multi-species study all animals caught in the CSA also had a higher $\delta^{15}N$ than the individuals of the same species in the NM. Therefore, there is enrichment in ^{15}N in the CSA compared with the NM. The major reason for this is likely to be due to the impact of anthropogenic inputs of nitrogen in the CSA. As mentioned in Chapter 1, the nitrate levels in the CSA have been known to be high for many years. The nitrogen input to the CSA is currently 80 $kt.y^{-1}$, in comparison to <10 $kt.y^{-1}$ in the NM (Baxter *et al.*, 2008).

The introduction of nitrates into a marine system from human and/or animal waste can cause the sea water to have $\delta^{15}N$ values of around 10‰ - 20‰ compared with water containing naturally-occurring nitrate deposited from the atmosphere, with a $\delta^{15}N$ value of between 2‰ - 8‰ (Bannon & Roman, 2008; Kendal, 2007). Kendal (2007) stated that it was not possible to deduce the source of the nitrate from the $\delta^{15}N$ value alone. However, the dual isotopes of $\delta^{15}N$ and $\delta^{18}O$ could be used to determine the reason why the base $\delta^{15}N$ is significantly higher in the CSA than in the NM.

Carlier *et al.* (2008) studied the effect of nitrates in two lagoons along the French Mediterranean coast. The Lapalme lagoon had a total nitrogen input of 4 ton.y^{-1} and the Carnet lagoon had an input of 43.8 ton.y^{-1} of nitrogen. The Particulate Organic Matter (POM) from the Lapalme lagoon was 3.6‰ compared to the Carnet lagoon with 13.0‰. Therefore the $\Delta^{15}N_{Carnet\text{-}Lapden}$ had a value of 9.4‰. They also measured the $\delta^{15}N$ of various invertebrates, and of those that were measured in both areas (*Abra* [as *A. ovata*] *segmentum*, *Carcinus maenas* and *Hediste* [as *Nereis*] *diversicolor*).

Trophic baseline is therefore an important consideration when controlling for this type of site enrichment (Cabana & Rasmussen, 1996; Post, 2002; Jennings & Warr, 2003). Two approaches were used to try and characterise the trophic baseline of the two sites in this study. The first approach is the use of POM, which many authors, including Hill (2008), have used to establish the trophic level of *N. norvegicus* in the Irish Sea. However, this proved difficult in this study due to the practical limitations of preparing uncontaminated samples on board commercial fishing vessels. There was too much variation between samples (standard deviation of 1.4‰ in June) when the $\delta^{15}N$ was calculated.

The second method utilised a longer living animal with a fixed trophic level, as has been used in a number of other studies including, Cabana & Rasmussen, (1996), Post (2002) and Jennings & Warr (2003). Jennings & Warr (2003) utilised the queen scallop, *A. opercularis*, to determine the trophic base line, setting the TL at 2.5 for this filter feeding species. The practical benefit of using this species is that the adductor muscle can be quickly frozen along with *N. norvegicus* samples. The preservation of POM requires rapid filtration to prevent live phytoplankton from being consumed by zooplankton (which was smaller than 250 µm diameter). In the filter feeding *A. opercularis*, its muscle tissue is laid down over an extended time period, therefore, the $\delta^{15}N$ value of this tissue will be time averaged for its trophic level, making it more reliable as a basis for calculating the baseline. The fact that the tissues of this animal average the local and temporal POM fluctuations makes it useful for baseline calculations (Jennings *et al.*, 2008).

Using the second method to determine the trophic baselines for the two sample locations, a value of 3.4‰ for the TEF was established from Equation 6.1. This suggests that *N. norvegicus* from the NM were feeding at an average trophic level of 3.7, which is 0.6 units higher than the animals from the CSA (3.1).

Table 6.4 shows other studies which measured δ^{15}N and δ^{13}C for *N. norvegicus* throughout its geographic range. Trophic level is shown when reported by the authors, or subsequently calculated when the δ^{15}N of POM or *A. opercularis* was also reported. This shows that the TL of *N. norvegicus* in the NM and the CSA calculated in the present study are within the range of other studies. The lowest TL for *N. norvegicus* was 2.7 calculated from the study by Sara *et al.* (2009) in Iceland. This was calculated via the POM δ^{15}N value of 5.2‰ reported in that paper. The highest reported trophic level for *N. norvegicus* of 4.3 was from the North Sea, as reported by Jennings *et al.* (2002). In that study the baseline for δ^{15}N was calculated from measurements of *A. opercularis*.

The ecological relevance of the difference between *N. norvegicus* populations is not yet clear, but should be addressed in future studies. One possible approach is to consider the types of food consumed by *N. norvegicus* at different sites, in order to seek evidence that higher trophic positioning may be related to a more pelagic-based diet. This approach has been followed to compare *N. norvegicus* between the CSA and the NM, using fatty acid analysis to obtain evidence about the food types consumed. The results of this work are reported in the following chapter (Chapter 7).

Table 6.4 Values of $\delta^{15}N$ and $\delta^{13}C$ from different *N. norvegicus* communities measured in various different studies, TL- Trophic level determined by the base reported; * Displayed in paper as 85.4 g, using the equation from Farmer (1974) 47 mm was estimated. ** indicates that TL was not calculated in the study but has been approximated relative to the base measure reported.

Site	**Size (CL)**	**$\delta^{15}N$ (‰)**	**$\delta^{13}C$ (‰)**	**TL**	**Base**	**Reference**
Iceland	?	11.1 ± 0.1	-16.2 ± 0.20	2.74**	POM (5.2‰ ± 0.5)	Sara *et al*. (2009)
North Minch	25-42 mm	10.98 ± 0.04	-16.40 ± 0.03	3.63	*A. opercularis* (7.19‰ ± 0.41)	this study
Clyde Sea Area	26-41 mm	12.96 ± 0.03	-15.55 ± 0.02	3.05	*A. opercularis* (10.92‰ ± 0.13)	this study
Irish Sea	25 mm	12.3 ± 0.03	-16.3 ± 0.04	2.60	POM (6.8‰ ± 0.03)	Hill (2008)
North Sea	~47 mm*	12.6 ± 0.24		4.32**	*A. opercularis* (6.4‰ ± 0.76)	Jennings *et al.* (2002)
Bay of Biscay	14-17 mm	10.68 ± 0.25	-17.55 ± 0.50	2.90	POM	Le Loc'h & Hily (2005)
	22-34 mm	11.04 ± 0.44	-16.51 ± 0.53	3.00	POM	
	39-42 mm	11.22 ± 0.76	-15.93 ± 0.25	3.10	POM	
Catalan sea	-	7.53 ± 0.32	-17.19 ± 0.20			Fanelli *et al.* (2011)

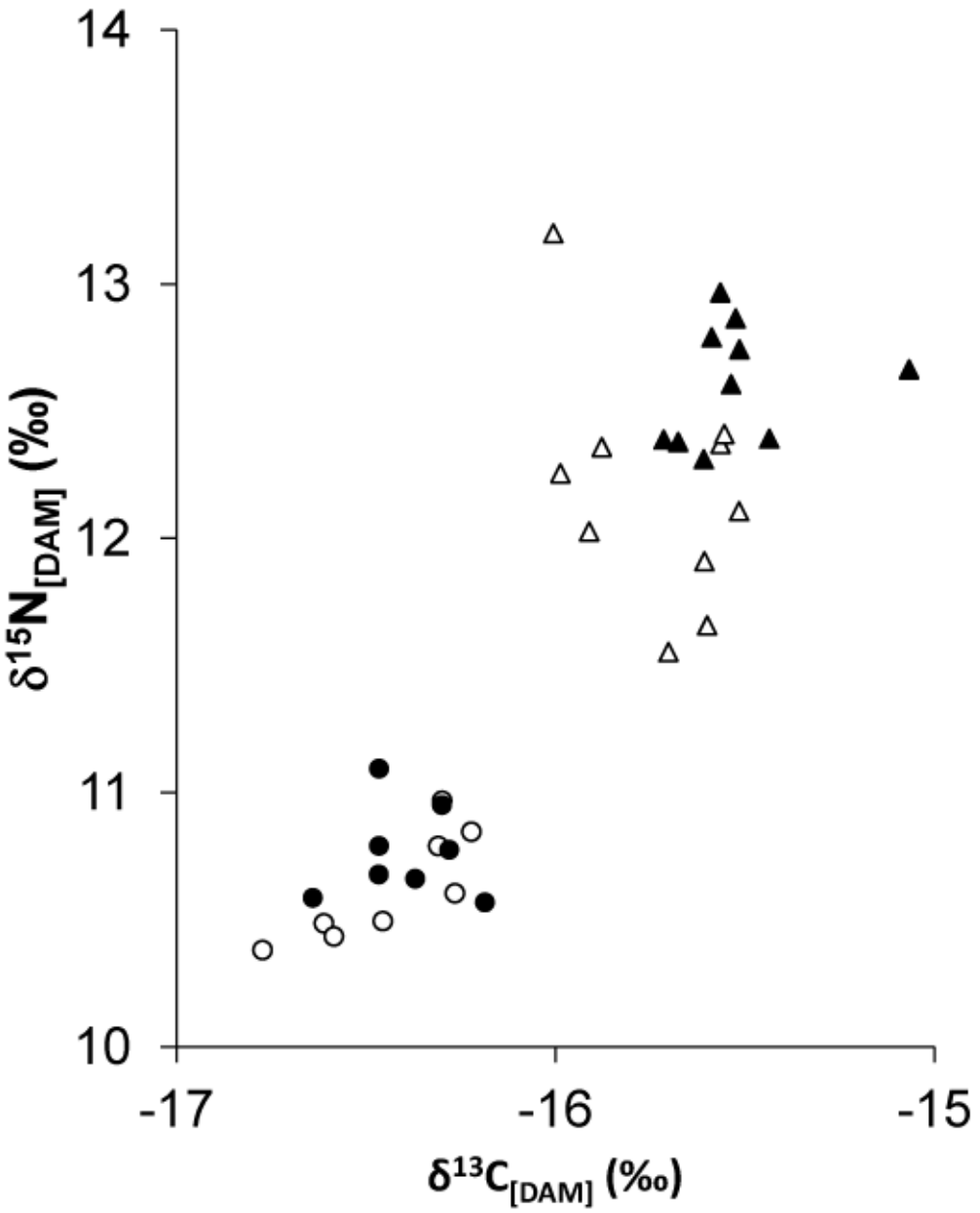

Figure 6.1 The stable isotopes of $\delta^{15}N$ and $\delta^{13}C$ in the tail muscle of male *N. norvegicus*, sampled from the Clyde Sea Area (CSA): north transect (black triangles) and west transect (white triangles); and from the North Minch (NM): east transect (black circles) and south transect (white circles). n=36

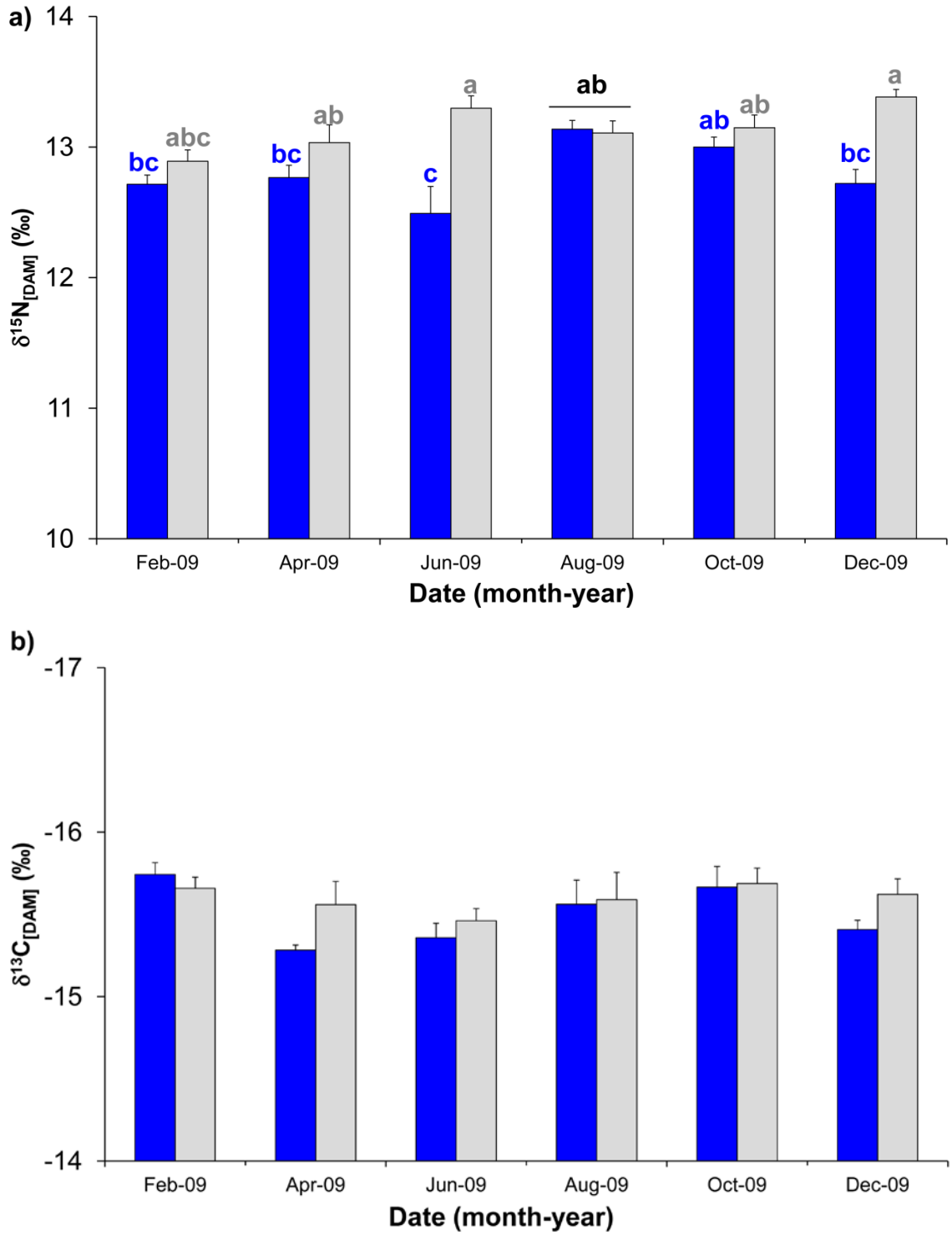

Figure 6.2 The stable isotopes of a) $\delta^{15}N$ and b) $\delta^{13}C$ of the tail muscle of male (blue bars) and female (grey bars) *N. norvegicus*, sampled bi-monthly in 2009 from the CSA. Error bars represent one standard error. Means that do not share a letter are significantly different (P>0.05) according to a post hoc Tukey test on the interaction of month and site. n=119

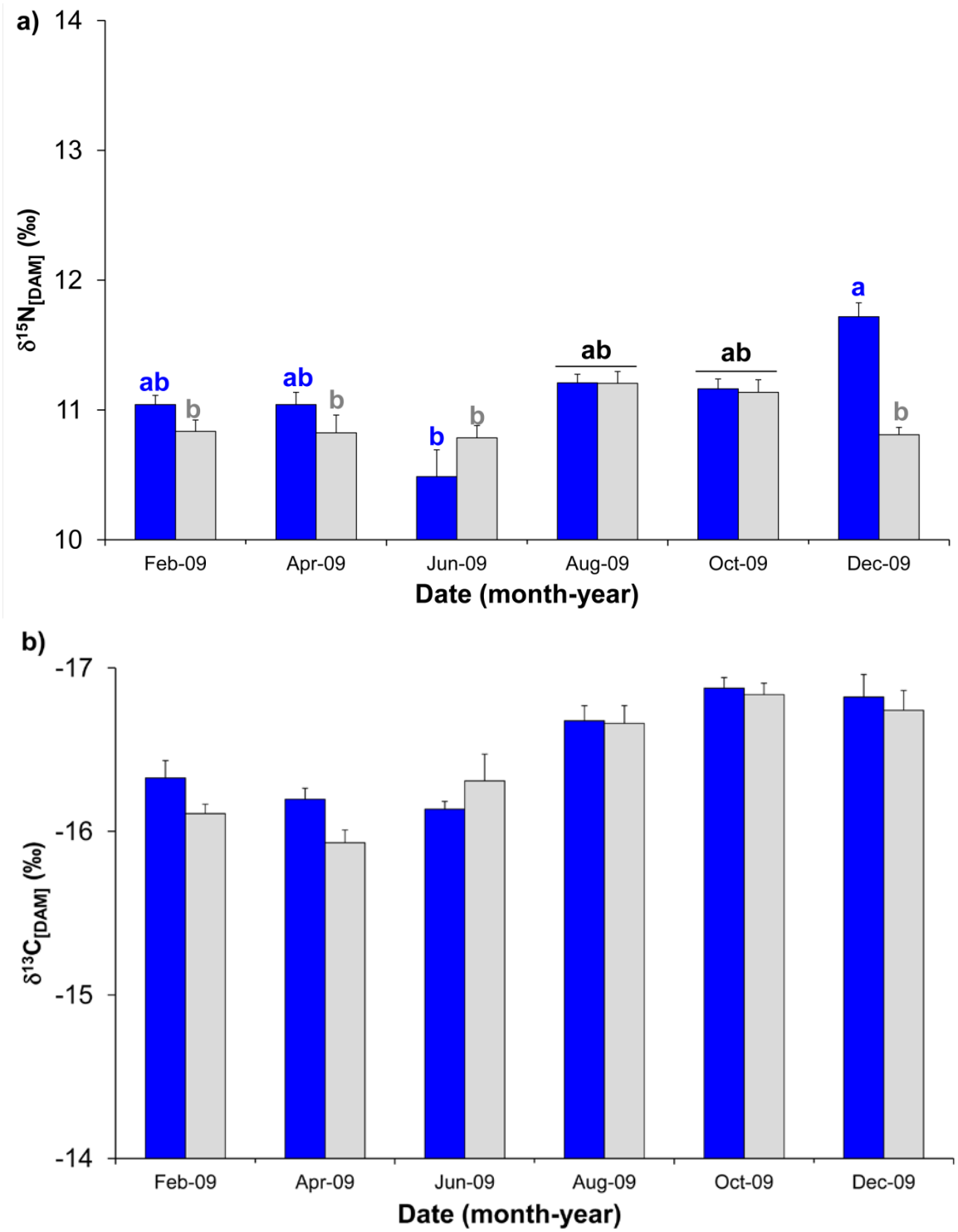

Figure 6.3 The stable isotopes of a) δ^{15}N and b) δ^{13}C of the tail muscle of male (blue bars) and female (grey bars) *N. norvegicus*, sampled bi-monthly in 2009 from the NM. Error bars represent one standard error. Means that do not share a letter are significantly different (P>0.05) according to a post hoc Tukey test on the interaction of month and site. n=120

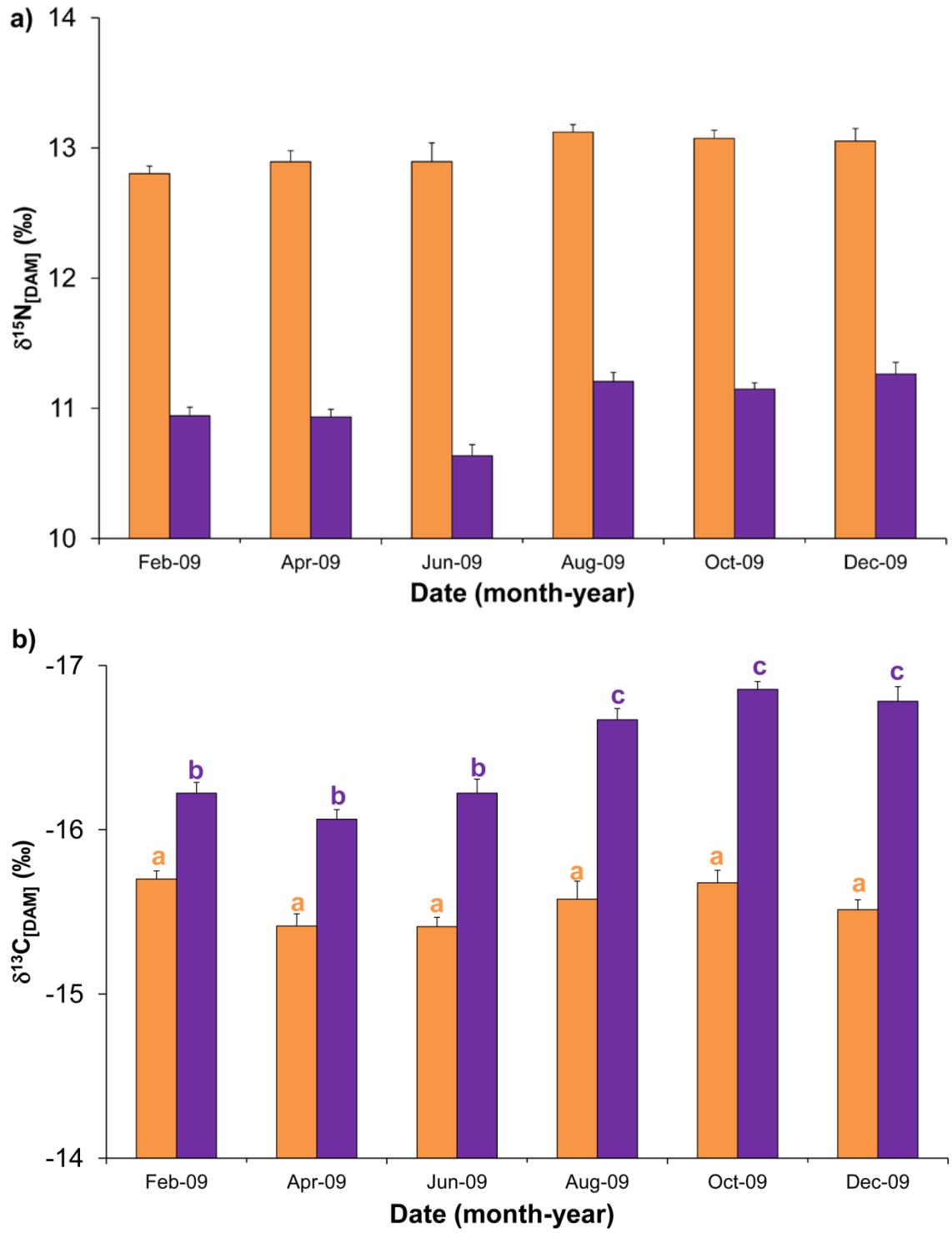

Figure 6.4 The stable isotopes of a) δ^{15}N and b) δ^{13}C of the tail muscle of *N. norvegicus*, sampled bi-monthly in 2009 from the CSA (orange bars) and NM (purple bars). Error bars represent one standard error. Means that do not share a letter are significantly different (P>0.05) according to a post hoc Tukey test on the interaction of month and site. n=239

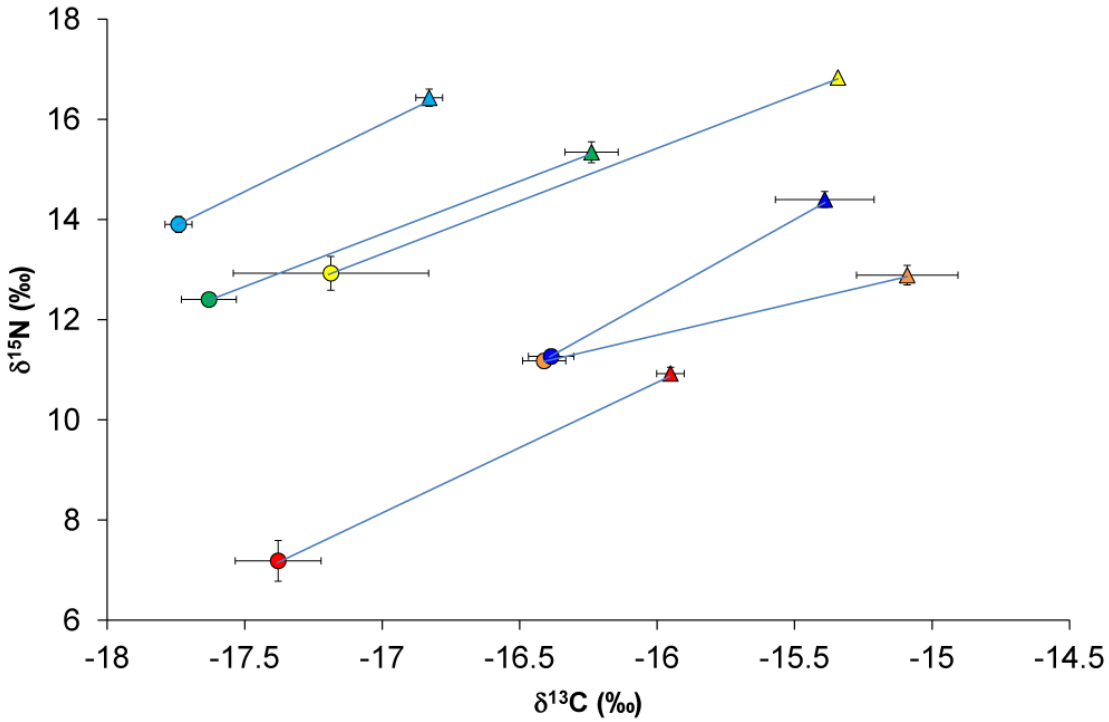

Figure 6.5 The stable isotopes of a) δ^{15}N and δ^{13}C of whiting, *Merlangius merlangus* (light blue), long rough dab, *Hippoglossoides platessoides* (green), lesser spotted dogfish, *Scyliorhinus canicula* (yellow), squat lobster, *Munida rugosa* (dark blue), Norway lobster, *Nephrops norvegicus* (orange), queen scallop, *Aequipecten opercularis* (red) caught from the CSA (triangles) and the NM (circles) in June 2010. Error bars are ± SE. Lines shows the link to the same species at different sites, rather than inferring a gradient of delta values between them. n=68

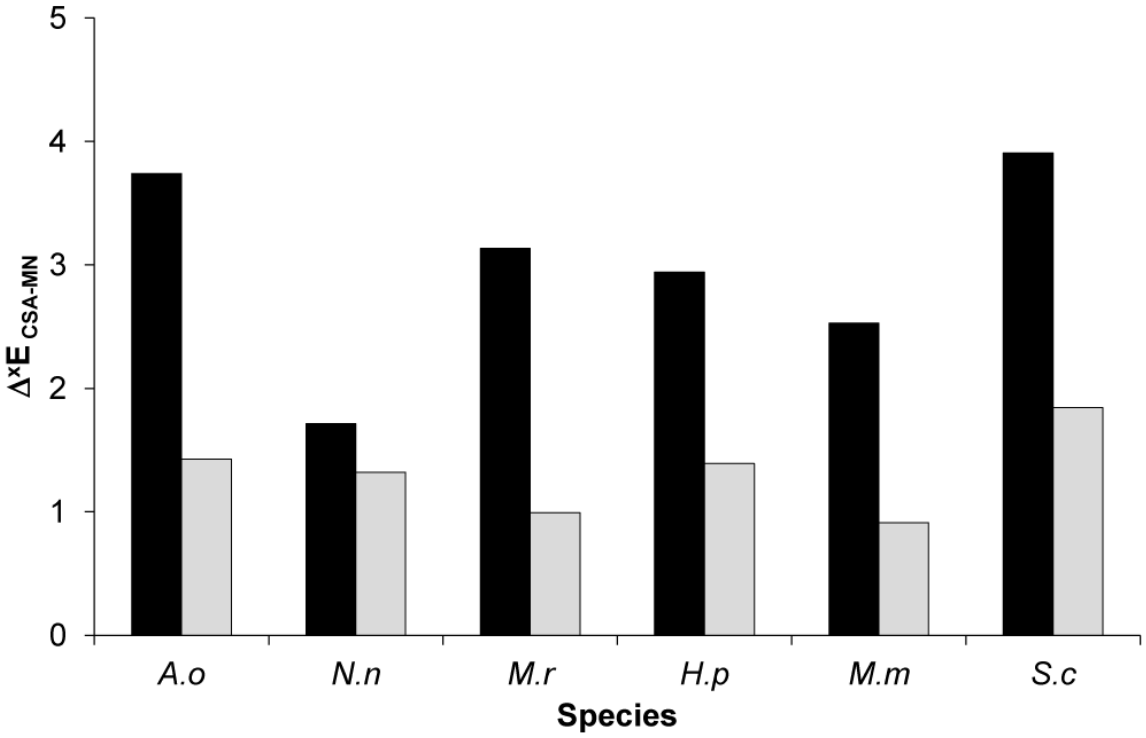

Figure 6.6 The mean difference (Δ) between the CSA and NM in $\delta^{15}N$ (black bars) and $\delta^{13}C$ (grey bars) of *A. opercularis* (*A.o*), *N. norvegicus* (*N.n*), *M. rugosa* (*M.r*), *H. platessoides* (*H.p*), *M. merlangus* (*M.m*) and *S. canicula* (*S.c*). n=68

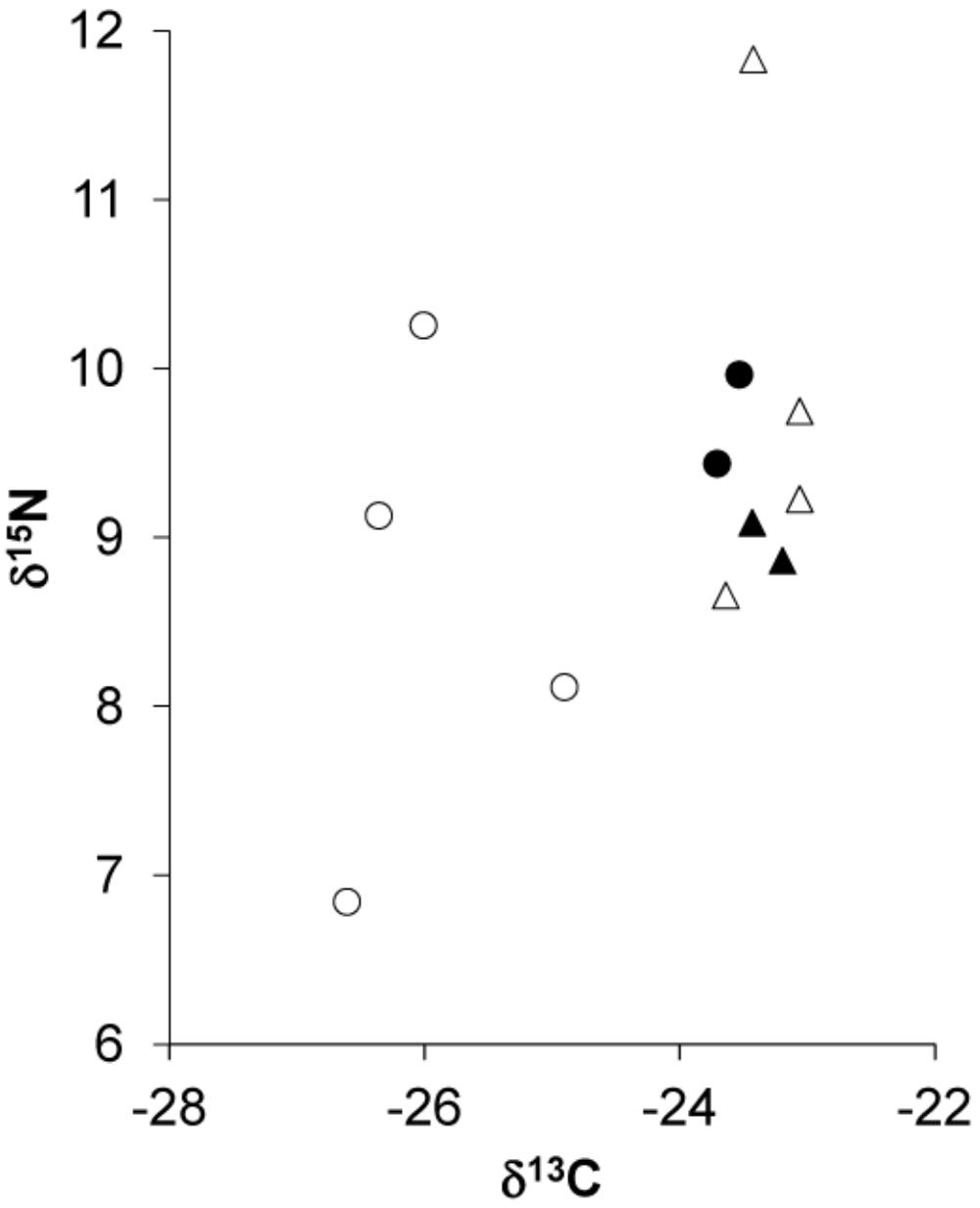

Figure 6.7 The stable isotope of δ^{15}N and δ^{13}C of particulate organic matter (POM). Data collected from the CSA (triangles) and the NM (circles) in June 2009 (black) and October 2009 (white). n=12

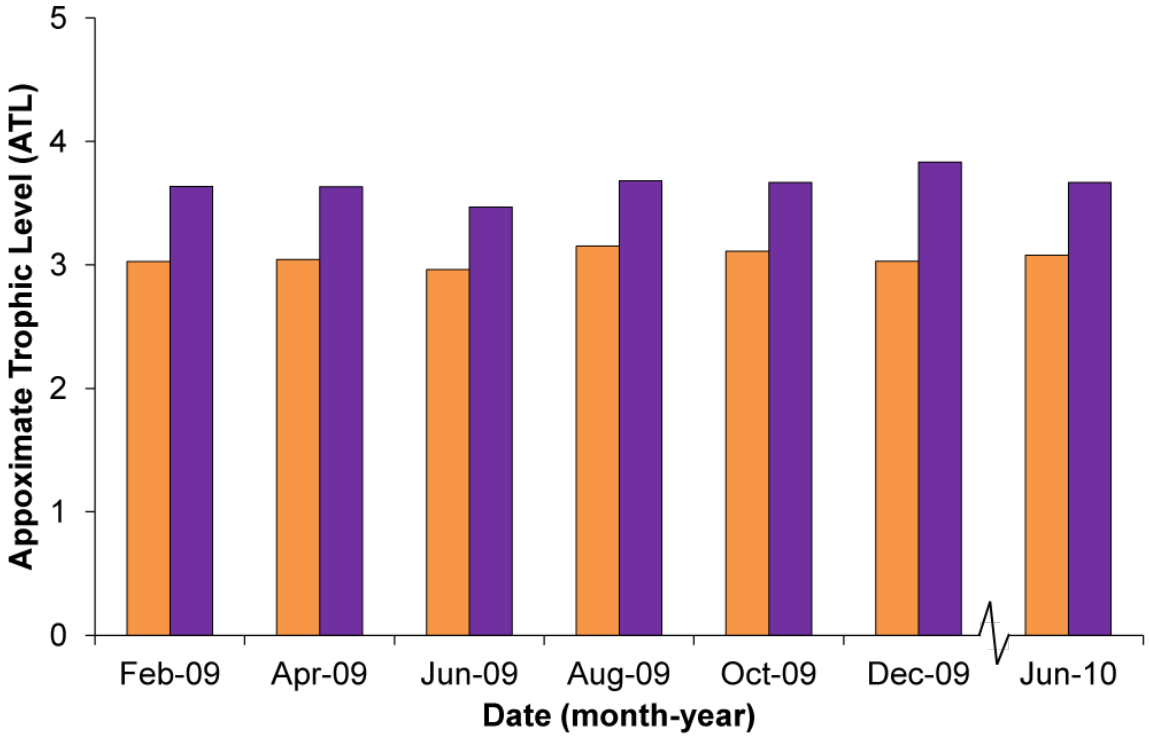

Figure 6.8 The mean of the approximate trophic level of male *N. norvegicus* from the CSA (orange bars) and NM (purple bars) sampled in 2009. n=289

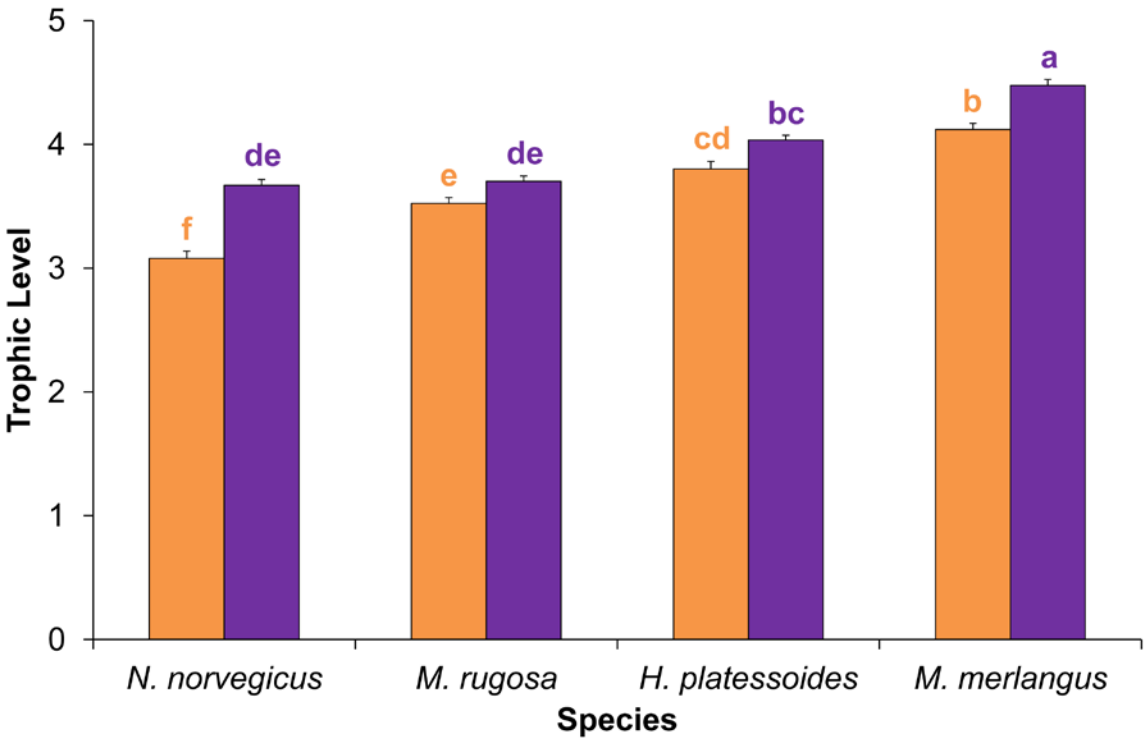

Figure 6.9 The mean (± SE) of the approximate trophic level (ATL) of various species from the CSA (orange bars) and NM (purple bars) sampled in 2009. Error bars represent one standard error. Means that do not share a letter are significantly different (P>0.05) according to a post hoc Tukey test on the interaction of month and site.

7 Trophic dynamics of *Nephrops norvegicus* off the west coast of Scotland (II): Fatty acid analysis

7.1 Introduction

7.1.1 Fatty acid analysis

Fatty acids (FAs) are neutral lipids which are subject to dietary turnover. Fatty Acid Analysis (FAA) has been used in nutritional studies for many years. Notable early work was carried out by Ackman & Eaton (1967), and then later by Christie (1982), who established this as a valid analysis for determining trophic dynamic interactions. Unlike gut contents analysis (GCA), FAA can provide information about assimilation (and not just consumption) of food and how it relates to long term dietary trends (Budge *et al.*, 2006; Stowasser *et al.*, 2009), and will not be biased by the presence in the stomach of hard structures which take time to digest.

Nomenclature of fatty acids

The nomenclature of fatty acids used in the present study is based on the system described by Christie (1982). The basic notation can be summarised as C:bn-x where 'C' is the number of carbon atoms in the fatty acid chain, 'b' is the number of double bonds between carbon atoms, and 'x' is the position of the first double bond as number of carbon atoms (or the number of carbon-carbon bonds) from the methyl group.

The abundance and distribution of the types and amounts of FA within a sample will from now on be referred to as FA signatures.

Families of fatty acids

Fatty acids are grouped into different families, based mainly on the number of double bonds contained within their structure. The three main families of FA are: saturated FAs (SFA), which contain no double bonds, e.g. 14:0; monounsaturated FAs (MUFA), also known as monoenoic acids, which contain one double bond, e.g. 16:1n-9; and polyunsaturated FAs (PUFA), which contain more than one double bond. PUFA can be subdivided according to the number of double bonds, e.g. PUFA-3 (18:4n-3) also known as Omega 3, and PUFA 6 (20:4n-6) also known as Omega-6. The structures of these FAs is shown in Figure 7.1

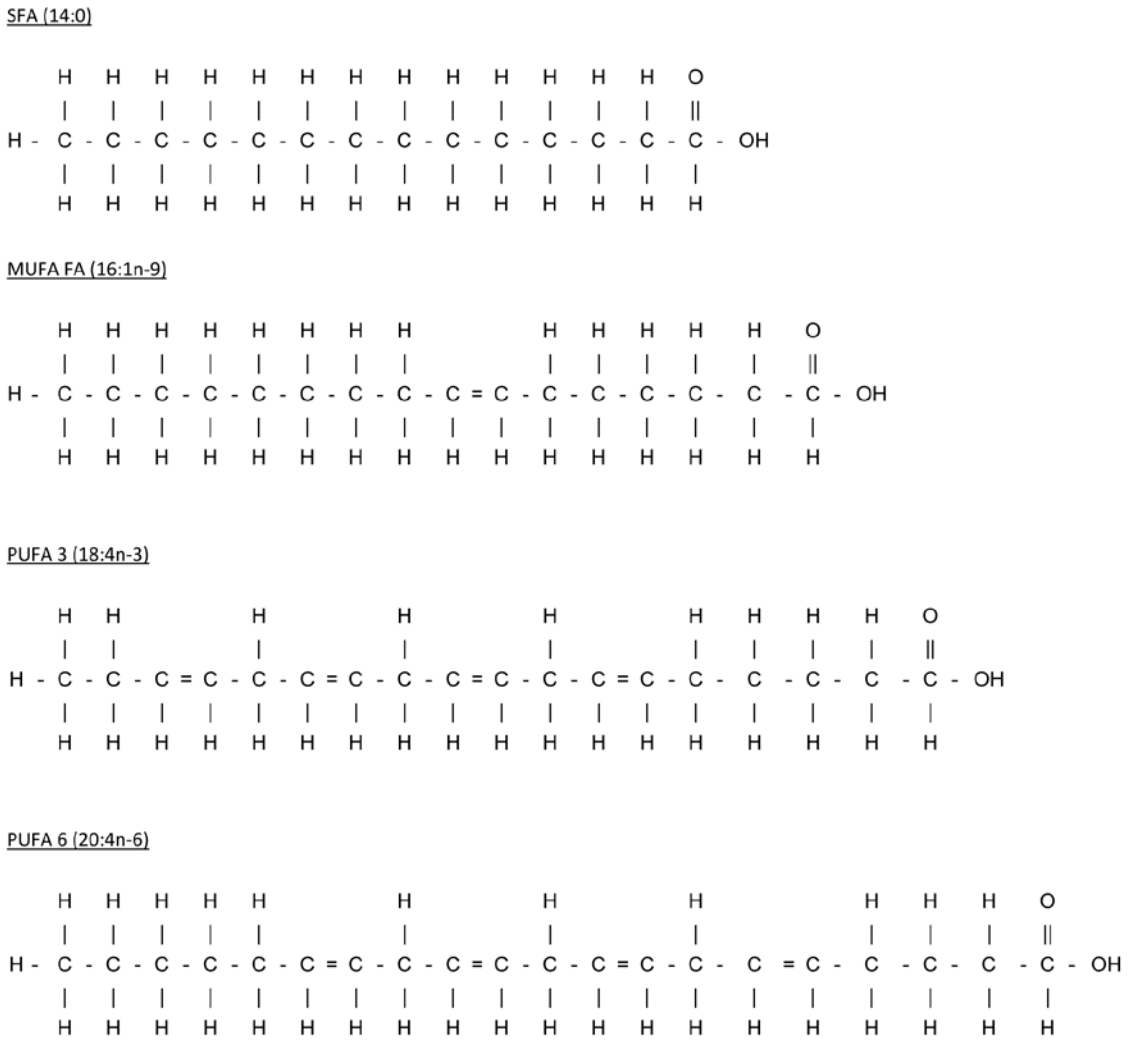

Figure 7.1 Chemical structure of four different Fatty Acids (FA): saturated fatty acids (SFA) contain no double bonds between carbon atoms; monounsaturated fatty acids (MUFA) contain one double bond between carbon atoms; polyunsaturated fatty acids (PUFA) contain a number of double bonds.

Other notable FAs are 'Iso' FAs, also known as branch chain fatty acids, as they contain a branched CH_3 on the last-but-one carbon atom. Antieiso FAs, in contrast, are branched chain fatty acids in which the CH_3 branches from the last-but-two carbon atom. Dimethyl aldehyde FAs (DMA), have two terminal CH_3 branches. Non-methylene-interrupted dienoic FAs (NMID) have traditionally been found in bivalve molluscs (Joseph, 1982; Budge *et al.*, 2006).

Food item identification through FA analysis

Animals receive a considerable proportion of their FAs from their diet (Sargent & Whittle, 1981). There are three ways in which these FAs are assimilated into tissues of a predator from its prey. Firstly, FAs may be derived directly and without modification from the diet; secondly, FAs may be derived from the diet but then modified by digestive processes; thirdly, FAs may be derived by *de novo* synthesis from endogenous sources. Within the tissues of an animal any FA that has a carbon chain length of less than 14 will probably have been synthesised *de novo*, since if ingested from prey such FAs would normally be oxidised by the digestive processes (Budge *et al.*, 2006).

Since many FAs originate mainly from the diet, knowledge of the fatty acid composition of different food items will make it possible to identify the food consumed over a particular period of time. For example, in the dominant hydrothermal shrimp species *Rimicaris exoculata* and fish species *Thermarces cerberus*, the amounts of different types of FA have been shown to reflect those of their prey species (Pond *et al.*, 2000, 2008). Thus if, under controlled captive conditions, individuals of a given species are fed on a particular prey, and their FA profiles are subsequently established, then wild-caught individuals of the same species displaying a similar profile could be assumed, with some confidence, to have been feeding on this same prey. However, under natural conditions *Nephrops norvegicus* will feed on a variety of food items, as shown by Yonge (1924), Thomas & Davidson (1962) and Parslow-Williams (1998). Therefore, realistically, the possibility of identifying dietary components to a species level will be very low, although a guide to the type of prey items consumed could possibly be obtained.

As explained in Chapter 1, Tuck *et al.* (1997a) suggested that the growth rate of *N. norvegicus* is density-dependent and also related to food availability. Parslow-

Williams (1998) showed that, in the Clyde Sea Area (CSA), coarse muds tend to be occupied by animals of a smaller average size, compared with those on finer muds and again related this to differences in food availability on these two sediment types in different areas. These factors may influence FA signatures and therefore may need to be considered.

7.1.2 Aim

Budge *et al.* (2006) describe three ways in which fatty acid analysis can be used to study the foraging ecology of a particular species, and the food web it occupies within an ecosystem. It can be used to:

1. Examine the changes in FA signatures that occur as a consequence of spatial and/or temporal variations in diet.
2. Identify unique FAs found in a predator that can be traced to a single origin or prey species. This is rare, due to the multiple inputs of prey items and relatively few instances in which there are unique FAs that can be used to identify the types of prey consumed.
3. Quantitatively estimate diet from FA signatures of predator and prey using computer models such as QFASA, as discussed by Iverson *et al.* (2003), to compare the most likely trophic relationships.

The present study will consider the first two of these approaches in relation to the feeding of *N. norvegicus* from two sites, the North Minch (NM) and the Clyde Sea Area (CSA), and will discuss the third approach in terms of both its statistical and biological significance. Since it is known that the hepatopancreas retains diet-sourced lipids for around three weeks before they are completely metabolised (Yonge, 1924), this organ was chosen for this study.

7.2 Methodology

7.2.1 Sites

N. norvegicus were sampled from the same two sites as described in previous chapters, namely the NM and CSA. The locations of these transects can be seen in Figure 1.5.

7.2.2 Samples used

A total of 72 *N. norvegicus* individuals were used in this study, comprising 3 males and 3 females collected from the main catches taken at the two sites bimonthly (from February to December 2009). All animals were in the intermoult stage, and were between 26.0 and 46.1mm carapace length. The males were significantly larger (36.35 ± 0.68 mm CL) than the females (33.50mm ± 0.62 CL) ($F_{1,67}$=10.28, P=0.002), but there was no significant difference in the size of the animals with regard to site ($F_{1,67}$= 2.55, P=0.155) or month ($F_{5,67}$=1.13, P=0.354).

The same collection method was used as outlined in Section 5.2.1.2 (Chapter 5), with the hepatopancreas being stored at -80˚C prior to freeze-drying and stored at -20˚C until lipid analysis was undertaken.

7.2.3 Fatty acid preparation

Lipid extractions were carried out on the hepatopancreas samples from groups of 12 animals at a time, derived from one male and one female from three of the bimonthly sample groups from both the NM and the CSA. The samples were then analysed in a random order (randomisation being carried out by assigning a random number, from Microsoft Excel) to each sample and then ranking by random number.

The total lipid fraction was prepared from the freeze-dried hepatopancreas tissues of the individual animals according to methods described by Folch *et al.* (1957) and was quantified gravimetrically as described in Chapter 2, Section 2.2. This fraction was held in a concentration of 10 $mg.ml^{-1}$ of chloroform:methanol

(2:1 v:v) + butylated hydroxytoluene (0.01%) (BHT) and stored at -20˚C. A 100 µl volume of the suspended lipid was placed in a small 'Quickfit' tube, and to this was added 100 µl of 17:0 free fatty acid standard (1:1 w:v FA standard : C:M (2:1 v:v)). This was evaporated to dryness under oxygen-free nitrogen (OFN) at 25˚C, and a volume of 1 ml of toluene was then added to ensure that the neutral lipids were dissolved. After adding 2 ml of methylation reagent (concentrated sulphuric acid : methanol (1% v/v), the mixture was vortexed, and then flushed with OFN, stoppered (with paper inserted to relieve pressure) and incubated at 50˚C for 16 h. During this time acid-catalyzed transmethylation occurred.

Fatty acid methyl esters (FAMEs) were extracted and purified as described previously by Tocher & Harvie (1988). Tubes were then cooled to room temperature and 2 ml of 2% $KHCO_3$ was added, along with 5 ml of iso-hexane : diethyl ether (1:1 v/v) + 0.01% (w/v) BHT. This mixture was vortexed and then centrifuged at 400 ***g*** for 2 minutes. The upper organic layer was then transferred to clean tubes and a further 5 ml of iso-hexane : diethyl ether (1:1 v/v) without BHT was added to the original tubes, which were then vortexed and centrifuged as before. The upper layer was added to the first upper layer, and was then evaporated to dryness under OFN at 25˚C.

The dried samples were re-suspended in 100 µl of iso-hexane and these were loaded onto Thin Layer Chromatography (TLC) 20x20 cm plates with 20 mm gaps between each sample. The plates were then dipped in iso-hexane: diethyl ether: acetic acid (90:10:1 v/v) until the liquid front reached 20 mm from the top. The plate edges were then sprayed with 1% iodine in chloroform to visualise the FA bands impregnated in to the silica which covered the plates. The bands (not including the BHT band at the top) were then marked and scraped off the plate into a clean Quickfit tube. 1 ml iso-hexane: diethyl ether (1:1 v/v) with BHT and 9 ml iso-hexane: diethyl ether (1:1 v/v) without BHT was added to the impregnated silica and the tubes were vortexed and centrifuged. The solvent was then removed into clean tubes (not disturbing the silica) and evaporated to dryness again. A volume of 1 ml of iso-hexane was added to the sample, which was stored in a brown GC bottle under OFN. All samples prepared in this way were then stored at -20˚C.

7.2.4 Fatty acid GC

The samples were sent (in another random order) to the Institute of Aquaculture, University of Stirling, UK, to be processed by gas chromatography (GC). The fatty acids in each sample were separated and quantified in a GC (Fisons GC-8160, Thermo Scientific, Milan, Italy) equipped with a 30 mm x 0.32 mm i.d. x 0.25 µm ZB-wax column (Phenomenex, Cheshire, UK), fitted with 'on column' injection and flame ionization detection. Hydrogen was used as the carrier gas. The initial oven thermal gradient was at 40°C.min^{-1} from 50˚C to 150°C, followed by a gradient of 2°C.min^{-1} to a final temperature of 230°C. Individual FAMEs were identified by comparison with known standards (SupelcoT 37 FAME mix; Sigma-Aldrich Ltd., Poole, UK) and published data (Tocher & Harvie, 1988). Data were collected and processed using Chromcard for Windows Version 1.19 (Thermoquest Italia S.p.A., Milan, Italy). Additional peaks were identified by mass spectrometry on two samples. The area of a given peak (%) was then converted to a percentage of known peaks (*ca* 95% of all peaks). Peaks were identified in the order that they passed through the GC, and then the data were sorted into the actual biological groupings for statistical analysis.

7.2.5 Statistical analysis

FA values are displayed in the results tables as a proportion (%) of the total FAs in the sample.

FA signatures were analysed using PRIMER 5 software with FAs classified as variables and the hepatopancreas tissues from different individuals classified as samples. Site, sex, month and various combinations of the three were added as factors. A Bray-Crutis similarity with a square root transformation was first performed. From the resulting values, Multi Dimensional Scaling (MDS) plots were produced for various factors and sampling points. A One Way Analysis of Similarity (ANOSIM) with 999 maximum permutations was performed for each MDS plot produced. A multivariate pairwise comparison was also performed to assess the dissimilarities among levels of various factors, which were performed on square root transformed data. The R-statistic is the proportional difference between the mean rank similarity between each factor and the mean rank

similarity within sites. R = 1 when all replicates within a factor are more similar to each other than any replicates from different factors. R is zero when the mean rank similarities between and within sites are the same. The FAs were then ranked via a SIMPER test according to how much weight they added to the difference between factors.

Family groups of FAs (saturates, monounsaturates, polyunsaturates, etc.), as well as particular FAs identified by SIMPER analyses as contributing to the dissimilarity between sites, were analysed using General Linear Models (GLM) in Minitab 16.

Total number of observations in each table and figure where appropriate are reported as n=# or (when statistical output is present in tables) n=total df + 1, a breakdown of numbers of observations in each factor is displayed within the appendices.

The samples from the CSA for April were subject to oxidation due to improper storage (Figure 7.2), leading to loss of the PUFA components. These data were therefore removed from all multivariate analyses.

7.3 Results

7.3.1 Factors that explain the greatest difference in individual fatty acids and their distribution

The concentration of each FA for each month is shown in Table 7.1 for males and in Table 7.2 for females.

Table 7.1 The fatty acid concentrations (%) in the hepatopancreas of male *N. norvegicus*, averaged over the number of animals in each group (N). *CSA samples from April 2009 were removed from the analyses due to oxidation during storage. n=31

Male *N. norvegicus*

Site	Clyde					Minch					
Month	Feb-09	Jun-09	Aug-09	Oct-09	Dec-09	Feb-09	Apr-09	Jun-09	Aug-09	Oct-09	Dec-09
N	3	3	3	3	3	2	3	3	3	2	3
14:0	1.37	1.94	1.65	1.77	1.55	2.19	1.28	2.09	2.19	2.49	2.31
anteiso 15:0	0.45	0.24	0.25	0.24	0.33	0.56	0.55	0.43	0.42	0.37	0.40
iso 15:0	0.27	0.15	0.11	0.10	0.18	0.26	0.30	0.18	0.20	0.14	0.13
15:0	1.09	0.64	0.55	0.62	0.64	0.72	0.58	0.54	0.57	0.55	0.47
iso 16:0	0.60	0.29	0.30	0.27	0.35	0.52	0.51	0.31	0.29	0.27	0.25
16:0	13.57	12.48	12.46	12.41	13.59	16.03	12.28	14.55	14.87	12.28	13.32
anteiso 17:0	1.48	0.94	0.95	0.83	1.06	1.14	1.21	0.84	0.76	0.74	0.67
iso 17:0	1.38	0.61	0.76	0.68	0.84	0.90	1.19	0.63	0.58	0.46	0.44
iso 18:0	0.97	0.51	0.53	0.55	0.68	0.84	0.76	0.52	0.59	0.34	0.34
18:0	5.55	4.86	4.35	4.53	4.69	4.68	4.35	4.29	3.22	2.87	3.04
iso 19:0	0.70	0.46	0.38	0.47	0.57	0.22	0.38	0.43	0.17	0.12	0.10
19:0	0.36	0.25	0.25	0.27	0.31	0.28	0.31	0.26	0.21	0.20	0.18
20:0	0.67	0.35	0.41	0.47	0.54	0.52	0.46	0.38	0.31	0.37	0.32
22:0	0.35	0.15	0.20	0.18	0.24	0.17	0.45	0.36	0.44	0.24	0.17
16:1n-9	0.27	0.22	0.32	0.30	0.32	0.31	0.33	0.33	0.29	0.27	0.29
16:1n-7	5.54	8.23	7.13	6.98	6.52	4.31	3.80	5.95	5.53	4.52	4.96
17:1	0.90	0.51	0.52	0.62	0.62	0.73	0.84	0.64	0.66	0.55	0.54
18:1n-9	14.83	10.97	12.75	12.57	14.26	15.17	14.22	15.70	15.65	12.26	14.43
18:1n-7	9.04	7.85	8.99	8.47	9.41	6.72	7.43	7.21	6.84	4.72	5.03
19:1	0.42	0.55	0.39	0.48	0.45	0.23	0.32	0.30	0.26	0.20	0.18
20:1n-11	6.30	4.12	4.15	4.85	4.76	3.58	2.91	1.79	1.78	2.61	2.31
20:1n-9	4.95	2.66	2.92	2.72	3.33	9.40	5.26	4.22	5.59	8.98	8.59
20:1n-7	8.33	5.69	5.68	5.95	6.29	1.96	2.67	1.81	1.68	1.66	1.19
22:1n-11	2.21	0.39	0.34	0.32	0.59	12.10	5.40	3.22	5.22	12.86	11.13
22:1n-9	1.12	0.60	0.73	0.63	0.83	1.20	0.93	0.83	1.16	1.36	1.32
22:1n-7	2.05	1.12	1.69	1.33	1.64	0.69	0.88	0.73	0.74	0.57	0.52
24:1n-9	0.28	0.08	0.00	0.11	0.17	0.95	0.81	0.83	0.56	0.91	0.93
18:2n-6	0.78	0.62	0.71	0.68	0.72	0.73	0.71	0.45	0.89	0.83	0.83
18:3n-6	0.04	0.13	0.04	0.13	0.09	0.00	0.05	0.11	0.08	0.12	0.11
20:2n-6	1.46	1.15	1.42	1.13	1.27	0.96	1.45	1.06	1.09	0.88	0.73
20:3n-6	0.12	0.43	0.34	0.47	0.44	0.00	0.15	0.08	0.07	0.05	0.05
20:4n-6	2.68	3.47	3.74	3.80	2.78	1.28	2.98	2.61	2.28	1.59	1.40
22:4n-6	0.55	0.72	0.90	0.88	0.86	0.33	0.77	0.47	0.45	0.50	0.37
22:5n-6	0.37	0.33	0.37	0.35	0.32	0.15	0.38	0.31	0.30	0.32	0.27
18:3n-3	0.24	0.18	0.17	0.21	0.27	0.33	0.31	0.37	0.50	0.60	0.50
18:4n-3	0.10	0.78	0.34	0.61	0.49	0.21	0.19	0.68	0.58	0.77	0.69
20:3n-3	0.15	0.11	0.19	0.15	0.18	0.23	0.28	0.28	0.36	0.34	0.27
20:4n-3	0.12	0.47	0.33	0.38	0.35	0.28	0.38	0.49	0.53	0.60	0.56
20:5n-3	3.36	14.96	13.44	14.22	9.46	2.60	6.85	9.72	9.30	6.93	6.81
22:5n-3	0.79	2.07	2.13	2.05	1.82	0.61	1.47	1.38	1.21	1.39	1.33
22:6n-3	2.85	5.97	6.13	5.03	5.15	4.23	10.89	10.62	10.23	10.54	10.92
16:2	0.07	0.51	0.31	0.29	0.21	0.10	0.08	0.24	0.17	0.20	0.20
16:3	0.00	0.25	0.04	0.15	0.00	0.00	0.00	0.06	0.03	0.03	0.07
16:4	0.00	0.11	0.13	0.19	0.06	0.00	0.00	0.19	0.32	0.05	0.03
16:0 DMA	0.33	0.21	0.19	0.13	0.19	0.27	0.60	0.38	0.35	0.26	0.25
18:0 DMA	0.36	0.30	0.17	0.12	0.21	0.27	0.56	0.27	0.22	0.21	0.21
18:1 DMA	0.04	0.13	0.00	0.03	0.00	0.04	0.18	0.10	0.00	0.04	0.05
22:0 NMID	0.57	0.25	0.17	0.31	0.39	1.03	1.32	0.78	0.48	0.83	0.77

Table 7.2 The fatty acid concentrations (%) in the hepatopancreas of female *N. norvegicus*, averaged over the number of animals in each group (N). *CSA samples from April 2009 were removed from the analyses due to oxidation during storage. n=31

Female *N. norvegicus*

Site	Clyde					Minch					
Month	Feb-09	Jun-09	Aug-09	Oct-09	Dec-09	Feb-09	Apr-09	Jun-09	Aug-09	Oct-09	Dec-09
n	3	3	3	2	2	3	3	3	3	3	3
14:0	1.35	2.61	2.44	2.05	1.98	2.38	2.15	2.73	3.40	2.85	3.34
anteiso 15:0	0.37	0.25	0.30	0.35	0.35	0.60	0.70	0.98	0.57	0.39	0.34
iso 15:0	0.21	0.12	0.13	0.10	0.21	0.30	0.36	0.58	0.29	0.17	0.10
15:0	0.70	0.64	0.62	0.59	0.73	0.75	0.66	0.74	0.60	0.56	0.47
iso 16:0	0.41	0.28	0.27	0.29	0.37	0.50	0.56	0.55	0.35	0.35	0.15
16:0	13.38	12.46	12.43	13.62	14.14	13.88	14.64	14.62	13.13	11.91	12.11
anteiso 17:0	1.03	0.89	0.75	0.85	0.65	1.03	1.13	1.00	0.76	0.76	0.40
iso 17:0	0.93	0.70	0.62	0.69	0.81	0.81	0.96	0.81	0.58	0.62	0.20
iso 18:0	0.62	0.45	0.30	0.25	0.32	0.72	0.80	0.59	0.53	0.47	0.35
18:0	3.64	4.84	3.99	4.10	3.63	2.35	3.56	3.32	2.93	2.72	2.48
iso 19:0	0.50	0.47	0.31	0.40	0.44	0.21	0.26	0.24	0.18	0.10	0.00
19:0	0.22	0.28	0.22	0.10	0.21	0.28	0.28	0.27	0.20	0.26	0.16
20:0	0.48	0.56	0.36	0.36	0.34	0.48	0.50	0.41	0.36	0.30	0.27
22:0	0.23	0.09	0.09	0.12	0.30	0.43	0.44	0.38	0.39	0.25	0.16
16:1n-9	0.27	0.29	0.32	0.27	0.36	0.42	0.33	0.42	0.35	0.31	0.26
16:1n-7	6.88	9.71	8.68	8.00	7.80	6.44	5.96	7.41	6.72	5.09	5.02
17:1	0.72	0.55	0.52	0.53	0.66	0.92	0.77	0.71	0.69	0.61	0.41
18:1n-9	16.27	14.23	12.12	13.24	13.88	15.22	15.17	14.80	15.69	11.94	12.99
18:1n-7	9.18	10.30	8.61	9.03	9.39	8.08	8.55	8.16	7.05	5.10	3.75
19:1	0.47	0.50	0.55	0.20	0.48	0.40	0.33	0.30	0.26	0.23	0.13
20:1n-11	5.43	5.60	4.34	4.64	4.52	2.40	2.35	2.83	2.11	1.90	2.48
20:1n-9	4.37	4.28	2.68	3.03	2.91	4.79	5.56	4.66	6.27	9.63	11.55
20:1n-7	6.86	8.11	5.48	5.56	5.47	2.23	2.51	2.06	1.84	1.97	0.99
22:1n-11	1.89	1.10	0.77	0.47	0.74	3.81	4.52	4.01	7.23	11.08	15.67
22:1n-9	0.97	1.06	0.64	0.64	0.86	0.92	1.26	1.15	1.22	1.19	1.50
22:1n-7	1.34	1.51	1.03	1.07	1.13	0.70	0.82	0.61	0.54	0.47	0.31
24:1n-9	0.14	0.03	0.14	0.00	0.00	0.41	0.55	0.64	0.50	0.48	0.78
18:2n-6	0.71	0.49	0.83	0.66	0.69	0.68	0.67	0.59	0.74	0.96	0.89
18:3n-6	0.14	0.08	0.14	0.05	0.16	0.13	0.03	0.13	0.11	0.15	0.10
20:2n-6	1.25	1.08	0.98	1.19	1.09	1.37	1.60	1.16	1.04	0.84	0.63
20:3n-6	0.20	0.20	0.33	0.30	0.40	0.11	0.07	0.02	0.05	0.48	0.07
20:4n-6	3.07	2.01	3.13	3.31	3.21	2.90	2.60	2.67	1.86	1.65	1.09
22:4n-6	0.65	0.34	0.77	0.85	0.79	0.79	0.65	0.60	0.46	0.56	0.24
22:5n-6	0.48	0.14	0.30	0.30	0.37	0.43	0.38	0.37	0.29	0.20	0.24
18:3n-3	0.24	0.09	0.26	0.10	0.21	0.43	0.35	0.41	0.53	0.82	0.71
18:4n-3	0.19	0.38	1.11	0.54	0.53	0.48	0.33	0.57	0.76	1.19	1.17
20:3n-3	0.22	0.11	0.16	0.06	0.17	0.37	0.36	0.30	0.36	0.32	0.29
20:4n-3	0.27	0.21	0.46	0.36	0.37	0.55	0.47	0.45	0.55	0.68	0.73
20:5n-3	6.70	8.58	13.64	13.95	11.69	7.85	6.58	6.85	7.80	7.87	6.25
22:5n-3	1.33	0.94	1.69	1.93	1.75	1.54	1.17	1.15	1.08	1.43	1.05
22:6n-3	4.66	2.26	5.86	4.72	4.88	8.71	7.41	7.50	7.97	9.53	9.21
16:2	0.16	0.37	0.48	0.34	0.29	0.12	0.12	0.12	0.22	0.25	0.27
16:3	0.04	0.00	0.37	0.07	0.09	0.00	0.00	0.08	0.04	0.08	0.11
16:4	0.00	0.21	0.35	0.17	0.24	0.00	0.00	0.11	0.14	0.10	0.06
16:0 DMA	0.19	0.09	0.14	0.08	0.17	0.36	0.43	0.49	0.32	0.29	0.10
18:0 DMA	0.24	0.09	0.06	0.07	0.08	0.31	0.34	0.25	0.12	0.18	0.05
18:1 DMA	0.03	0.00	0.00	0.00	0.00	0.06	0.09	0.10	0.02	0.04	0.15
22:0 NMID	0.39	0.41	0.26	0.38	0.15	1.32	0.64	1.11	0.81	0.66	0.34

Figure 7.3a shows the MDS plot for the factor 'site', which shows a clear dissimilarity between the groupings of the NM and the CSA. The ANOSIM results indicate that different sites cluster into a discrete group, (R=0.664, P=0.001). The factors 'sex' and 'month' were tested in each site separately.

Figure 7.3b shows the MDS plot with the factor 'sex', which shows that there is no clear difference between male and female groups, as confirmed by the ANOSIM results which indicate sexes do not cluster the animals from the NM (R=0.005, P=0.102) or the animals from the CSA (R=0.106, P=0.149).

Figure 7.3c shows the MDS plot with the factor 'month', which shows dissimilarity between the groupings of each month. The ANOSIM results indicate that the factor 'month' does significantly cluster in the NM (R=0.429, P=0.001) and in animals from the CSA (R=0.24, P=0.002).

There are, however, subdivisions within the month groups and so an ANOSIM with the combined factor of 'site and month' was calculated (Figure 7.3d). This indicated that there was significant variation in fatty acid profiles among month-site combinations (R=0.694, P=0.001) and pairwise tests indicated which months were most similar (Table 7.3). All animals at all times from the CSA were significantly different from those in the NM at equivalent sampling points.

Within sites, animals from the NM in February were not significantly different from those in April and June, but were significantly different from those in August, October and December. Animals from the CSA in February were significantly different from the animals caught in all other months, while those in June, August, October and December were not significantly different from each other.

Table 7.3 Pairwise comparison of FA signatures between Month and Site. R-statistic. Proportional difference between the mean rank similarity between site*month interaction and the mean rank similarity within sites. R = 1 when all replicates within the site month interaction are more similar to each other than any replicates from different sites.

		NM					CSA				
		Apr	**Jun**	**Aug**	**Oct**	**Dec**	**Feb**	**Jun**	**Aug**	**Oct**	**Dec**
NM	**Feb**	0.136	0.723	0.888	0.916	0.715	0.672	0.723	0.888	0.916	0.768
	Apr		0.226	0.617	0.472	0.802		0.78	0.967	1.00	0.981
	Jun			0.263	0.464	0.741		0.737	0.859	0.941	0.875
	Aug				0.565	0.669			0.904	1.00	0.987
	Oct					0.013				0.84	0.800
	Dec										0.987
CSA	**Feb**	0.824	0.833	0.956	0.776	0.98		0.496	0.717	0.765	0.419
	Jun			0.789	0.763	0.907			-0.96	-0.83	-0.64
	Aug				0.849	0.981				-0.77	-0.08
	Oct					0.995					0.124

7.3.2 Fatty acids related to site similarity

Animals selected and analysed from the NM had an average within-group similarity of 89.76%, a value slightly lower than that for the CSA (90.05%). The three FAs that contributed most to the similarly in site for the NM were 18:1n-9 (contributing 7.76% of the similarity), FA 16:0 (contributing 7.51% of the similarity) and FA 22:6n-3 (contributing 5.89% of the similarity). For the CSA the three FAs that contributed most to the similarly in site were FA 16:0 (contributing 7.42% of the similarity), FA 18:1n-9 (contributing 7.36% of the similarity) and FA 18:1n-7 (contributing 6.19% of the similarity).

7.3.3 Fatty acids related to site clustering

The extent to which different FAs contributed to site clustering between the NM and the CSA was determined by SIMPER analysis. Table 7.4 shows all of the FAs that contribute cumulatively to 90% of the dissimilarly between the NM and CSA sites.

The SIMPER analysis indicates that the abundance of FA 22:1n-11 contributed most (10.92%) to the dissimilarity between the two sites. This FA represented 7.76% of the total FAs in the hepatopancreas of animals from the NM, compared with a value of only 0.90% for animals from the CSA (Figure 7.4a).

The second most important contribution to dissimilarity between the two sites (7.13%) was made by FA 20:1n:7. This FA represented only 1.89% of the total FAs in the hepatopancreas of animals from the NM, compared with the higher value of 6.40% for animals from the CSA (Figure 7.4b).

The third most important contribution to dissimilarity between the two sites (5.55%) was made by FA 20:6n-3. This FA represented 9.07% of the total FAs in the hepatopancreas of animals from the NM, compared with 4.07% for animals from the CSA (Figure 7.4c).

Lastly, the FA 20:5n-3 contributed 5.37% to the dissimilarity between the two sites. This FA represented 7.26% of the total FAs in the hepatopancreas of animals from the NM and 10.87% for animals from the CSA (Figure 7.4d).

Table 7.4 SIMPER analysis on the dissimilarity of abundances of individual FAs between *N. norvegicus* from the NM and CSA.

Fatty acids	Average abundance		Average dissimilarity	Contribution (%)	
	NM	CSA		dissimilarity	Cumulative
22:1n-11	7.76	0.9	1.71	10.92	10.92
20:1n-7	1.89	6.4	1.12	7.13	18.04
22:6n-3	9.07	4.75	0.87	5.55	23.59
20:5n-3	7.26	10.87	0.84	5.37	28.97
20:1n-9	6.91	3.41	0.74	4.70	33.67
20:1n-11	2.38	4.89	0.67	4.27	37.94
24:1n-9	0.68	0.10	0.57	3.62	41.56
18:1n-7	6.60	9.01	0.46	2.91	44.47
16:1n-7	5.54	7.52	0.43	2.75	47.21
20:4n-6	2.11	3.11	0.39	2.51	49.72
22:1n-7	0.63	1.41	0.38	2.42	52.14
22:0 NMID	0.83	0.33	0.36	2.28	54.42
18:00	3.29	4.46	0.34	2.17	56.59
18:1n-9	14.48	13.51	0.34	2.14	58.73
20:3n-6	0.11	0.32	0.32	2.07	60.80
iso 19:0	0.20	0.47	0.31	1.98	62.77
18:4n-3	0.64	0.51	0.29	1.87	64.64
14:00	2.46	1.86	0.27	1.72	66.36
22:5n-3	1.25	1.64	0.26	1.65	68.01
18:3n-3	0.49	0.20	0.26	1.65	69.66
16:04	0.07	0.14	0.25	1.57	71.23
16:00	13.6	12.99	0.24	1.54	72.76
22:4n-6	0.52	0.72	0.23	1.44	74.20
22:00	0.33	0.19	0.22	1.41	75.61
iso 17:0	0.68	0.80	0.22	1.39	77.00
18:0 DMA	0.25	0.18	0.22	1.38	78.38
16:0 DMA	0.34	0.18	0.21	1.35	79.73
16:03	0.04	0.10	0.21	1.35	81.08
22:1n-9	1.16	0.81	0.20	1.29	82.38
iso 18:0	0.57	0.53	0.20	1.25	83.63
20:3n-3	0.31	0.15	0.19	1.22	84.85
20:4n-3	0.53	0.33	0.19	1.20	86.04
19:01	0.27	0.46	0.19	1.18	87.22
anteiso 17:0	0.87	0.96	0.18	1.17	88.39
16:02	0.18	0.30	0.18	1.16	89.55
18:1 DMA	0.06	0.02	0.18	1.12	90.67

7.3.4 Interaction of site and month in explaining the variation

Since the factor 'sex' was found not to explain any significant level of dissimilarity in the FA signature of *N. norvegicus*, only the interaction of month and site was considered in this section.

The four FAs that were determined to contribute to the dissimilarity of the FA signatures between each site (Table 7.4) were considered: 22:1n-11, 20:1n-7, 22:6n-3 and 20:5n-3. A GLM was constructed to test the null hypothesis that the factors 'month' and 'site' do not significantly explain the variation in the FAs with the model;

Fatty acid = month + site + month*site

The results are shown in Table 7.5 and Figure 7.5 to Figure 7.7. The samples from April for both sites were excluded because of degradation of those from the CSA during storage.

Figure 7.5 shows the variation of FA 22:1n-11 in *N. norvegicus* by month (in 2009) and by site. There was a significant effect of the interaction between site and month ($F_{4,55}$=8.96, P<0.001). The post hoc Tukey test (Table 7.6) indicated that the animals from the NM had a statistically greater mean proportion of 22:1n-11 in all months other than animals selected in February in the CSA (2.05 ± 0.15%) which was not significantly different to animals caught in the NM in June (3.62 ± 0.50%) or August (6.22 ± 0.63%), (Tukey, P<0.05). Within the NM there was a clear seasonal variation in the amounts of this FA, which were higher in the winter months than in the summer months.

Figure 7.6 shows the variation of FA 20:1n-7 in *N. norvegicus* by site and month during 2009. There was no significant effect of the interaction between site and month ($F_{4,55}$=1.32, P=0.278). However, there was a significant effect of site ($F_{1,55}$=280.46, P<0.001), where animals from the CSA (6.40 ± 0.27%) had statistically more of this FA compared with those from the NM (1.75 ± 0.14%).

Figure 7.7 shows the variation of FA 22:6n-3 in *N. norvegicus* by site and month during 2009. There was no significant effect of the interaction between site and

month ($F_{4,55}$=0.75, P=0.560). However, there was a significant effect of site ($F_{1,55}$=64.26, P<0.001), as with FA 22:1n-11. There was a relatively lower amount of this FA in animals from the CSA (4.77 ± 0.38%) compared with those from the NM (9.06 ± 0.41%).

Figure 7.8 shows the variation of FA 20:5n-3 in *N. norvegicus* by site and month during 2009. There was a significant effect of the interaction between site and month ($F_{4,55}$=3.16, P=0.022). The post hoc Tukey test (Table 7.6) was unable to separate any differences between sites in any month. However, annually there was a significant difference between sites ($F_{1,55}$=29.24, P<0.001) indicating that the animals in the CSA (4.75 ± 0.38%) have statistically more of this FA than animals in the NM (9.06 ± 0.41%).

Table 7.5 General Linear Model output for the FAs most abundant in the NM (by SIMPER) in terms of month, site and the interaction between month and site. Data for April for both sites omitted.

		22:1n-11		20:1n-7		22:6n-3		20:5n-3	
	df	F	P	F	P	F	P	F	P
Site	1,55	139.52	**<0.001**	280.46	**<0.001**	64.26	**<0.001**	29.24	**<0.001**
Month	4,55	7.64	**<0.001**	3.31	**0.018**	2.58	0.050	9.51	**<0.001**
Site * Month	4,55	8.96	**<0.001**	1.32	0.278	0.75	0.560	3.16	**0.022**

Table 7.6 The results of the post hoc Tukey test on the significant effect of the interaction between site and month for animals of 22:1n-11 and 20:5n-3. Bold letters signify non-significantly different groups of means. Letters for each FA values are different and cannot be cross-referred.

	22:1n-11		20:5n-3	
	CSA	NM	CSA	NM
Feb-09	2.05 ± 0.15 **de**	7.13 ± 2.08 **bc**	5.03 ± 1.11 **c**	5.75 ± 1.30 **abc**
Jun-09	0.75 ± 0.22 **e**	3.62 ± 0.50 **cde**	11.77 ± 1.86 **abc**	8.28 ± 0.88 **abc**
Aug-09	0.56 ± 0.16 **e**	6.22 ± 0.63 **cd**	13.54 ± 0.92 **ab**	8.55 ± 0.56 **bc**
Oct-09	0.38 ± 0.10 **e**	11.79 ± 1.88 **ab**	14.11 ± 1.00 **a**	7.49 ± 0.29 **abc**
Dec-09	0.65 ± 0.06 **e**	13.40 ± 1.65 **ab**	10.35 ± 1.15 **abc**	6.53 ± 0.50 **abc**

7.3.5 Differences in fatty acid families

The families of FAs were analysed to determine whether their variation can be explained by the factors 'site', 'month' or 'sex' and the results are summarised in Table 7.7.

The factor 'sex' does not significantly explain the variation in the families of saturated FAs (SFA) ($F_{1,55}$=1.75, P=0.191) or monounsaturated FAs (MUFA) ($F_{1,55}$=2.11, P=0.152), or in the polyunsaturated FAs PUFA n-6 ($F_{1,55}$=0.39, P=0.533), PUFA n-3 ($F_{1,55}$=0.32, P=0.575), the total PUFA ($F_{1,67}$=0.37, P=0.546) or the total DMA ($F_{1,67}$=2.74, P=0.104). Thus the factor of 'sex' was removed in further analyses. Since sex does not significantly explain variation in any FA family, data from the two sexes were combined for each month and for each site. A GLM was then applied to each grouping:

FA family = Site + Month + Site*Month

The variation in the GLM outputs for the different FA families are shown in Table 7.8.

There was a significant effect of the interaction of site and month on the proportion of SFA in *N. norvegicus* ($F_{4,55}$=3.27, P=0.019). The post hoc Tukey test (Table 7.9) indicated that there was no significant difference in the proportion of SFA between site in any month, but *N. norvegicus* caught in the NM in February (26.45 ± 0.87%) and June (26.51 ± 0.94%) have statistically more SFA than animals caught in December in the same site (21.35 ± 0.66%), (Tukey, P<0.05).

There was no significant effect of the interaction of site and month on the proportion of MUFA ($F_{4,55}$=2.54, P=0.053), Total PUFA ($F_{4,55}$=0.97, P=0.431) or Total DMA ($F_{4,55}$=1.07, P=0.384). There was also no significant difference between sites in MUFA ($F_{1,55}$=0.05, P=0.827) or Total PUFA ($F_{1,55}$=0.23, P=0.634). There was, however, a statistical difference in Total DMA between sites with the animals in the CSA having a mean percentage of 0.38 ± 0.04% which is statistically smaller than the relative proportion in the animals from NM (0.56 ± 0.06%).

Table 7.7 Totals amounts of hepatopancreas FA families for a) males and b) females. The amounts are averaged over the number of animals (N) in each group. *Data for CSA in April, which were excluded from the analyses. N=62

	CSA					NM					
Month	Feb-09	Jun-09	Aug-09	Oct-09	Dec-09	Feb-09	Apr-09	Jun-09	Aug-09	Oct-09	Dec-09
a) Male (n)	3	3	3	3	3	2	3	3	3	2	3
Total SFA	28.80	23.87	23.15	23.38	25.56	29.02	24.60	25.80	24.81	21.44	22.14
Total MUFA	56.23	43.00	45.61	45.32	49.19	57.35	45.79	43.55	45.96	51.49	51.44
Total n-6 PUFA	6.00	6.85	7.51	7.43	6.47	3.44	6.50	5.10	5.16	4.28	3.76
Total n-3 PUFA	7.61	24.54	22.73	22.65	17.72	8.48	20.37	23.53	22.71	21.18	21.09
Total PUFA	**13.61**	**31.38**	**30.24**	**30.08**	**24.19**	**11.92**	**26.87**	**28.63**	**27.87**	**25.46**	**24.84**
b) Female (n)	3	3	3	3	3	2	3	3	3	2	3
Total SFA	24.05	24.65	22.81	23.87	24.46	24.73	27.01	27.22	24.27	21.72	20.55
Total MUFA	54.79	57.26	45.88	46.69	48.21	46.75	48.69	47.74	50.47	50.00	55.83
Total n-6 PUFA	6.49	4.34	6.47	6.67	6.70	6.42	6.00	5.54	4.55	4.83	3.27
Total n-3 PUFA	13.61	12.57	23.19	21.66	19.60	19.93	16.68	17.23	19.05	21.84	19.42
Total PUFA	**20.11**	**16.91**	**29.66**	**28.34**	**26.30**	**26.35**	**22.68**	**22.78**	**23.60**	**26.67**	**22.68**

Table 7.8 GLM results analyse of how site, month and the interaction between these two factors explain the variation in different families of FA. All data for April from both sites excluded from the analysis.

		F	P	F	P	F	P
	df	SFA		MUFA		PUFA n-6	
Site	1,55	0.14	0.706	0.05	0.827	15.56	**<0.001**
Month	4,55	5.5	**0.001**	3.54	**0.013**	0.71	0.581
Site*month	4,55	3.27	**0.019**	2.54	0.053	1.61	0.189
		PUFA n-3		Total PUFA		Total DMA	
Site	1,55	0.66	0.420	0.23	0.634	7.7	**0.008**
Month	4,55	6.53	**<0.001**	5.35	**0.001**	3.4	**0.016**
Site*month	4,55	0.91	0.467	0.97	0.431	1.07	0.384

Table 7.9 The results of the post hoc Tukey test on the significant effect of the interaction between site and month for animals of saturated fatty acids. Bold letters signify non-significantly different groups of means. n=56

	SFA	
	CSA	NM
Feb-09	26.42 ± 1.47 **ab**	26.45 ± 0.87 **ab**
Jun-09	24.26 ± 0.81 **abc**	26.51 ± 0.94 **a**
Aug-09	22.98 ± 0.83 **abc**	24.54 ± 1.25 **abc**
Oct-09	23.19 ± 0.85 **abc**	21.99 ± 0.55 **bc**
Dec-09	25.12 ± 0.85 **abc**	21.35 ± 0.66 **c**

7.4 Discussion

The results obtained in this study demonstrate that there is a clear and sustained difference in the fatty acid signatures within the hepatopancreas of *N. norvegicus* from the Clyde Sea Area (CSA) compared with those from the North Minch (NM). It is known that this type of difference in the FA signatures of different individuals of one species indicates that these animals could be

consuming different prey species (Christie, 1982; Budge *et al.*, 2006). Although there was no clear difference in the FA families between the two sites studied, there was a clear difference in some changes by site and by month in individual FAs.

7.4.1 Individual fatty acids which explain site differences

The dissimilarities of FA signatures between animals from the two sites studied have been compared in order to identify the major contributing FAs.

22:1n-11 (Cetoleic acid)

The FA that accounted for the most dissimilarity between sites is FA 22:1n-11, also known as cetoleic acid. The animals from the NM had an average abundance of this FA of 7.76% (of all FAs identified), compared with 0.90% for the animals from the CSA. Cooper *et al.* (2006) noted that this FA varies widely among fish and invertebrates, and from other published literature, its source is likely to be calanoid copepods (Falk-Peterson *et al.*, 2000). Budge *et al.* (2002) showed that planktivores, such as herring and mackerel, which are pelagic feeders known to feed on these copepods, normally have a high abundance of this FA. Although there is no longer a commercial fishery for these species in the NM, mackerel, especially the horse mackerel *Trachurus trachurus*, are still present there, and represent one of the main bycatch species in *Nephrops* trawls (Milligan, unpublished data). Mackerel (*Scomber scombrus*) are also caught by recreational fishermen in the summer months. Therefore, there is a possibility of *T. trachurus* and other pelagic fish caught and discarded by trawlers entering the benthic food chain and thus the diet for *N. norvegicus* in the NM. There is, however, another possibility; dead copepods could be entering the benthic food chain, which are being consumed by marine invertebrates, and thus this difference could merely be a factor of depth (as the NM is much deeper than the CSA- see Section 5.2.1).

22:6n-3 (docosahexaenoic acid, DHA)

The FA 22:6n-3 (docosahexaenoic acid (DHA)) amounted to only 3.91% of the total FAs in the animals from the CSA, compared with more than double that value (9.07%) in the animals from the NM. Cook *et al.* (2010) and Kharlamenko *et al.* (1995) both suggested that the presence of this FA was evidence of

carnivorous feeding by marine invertebrates. Sargent & Whittle (1981) indicated that it was also a major FA of calanoid copepods. The results obtained by Budge *et al.* (2002) showed that a number of 'commercial' fish in Canada (that are still found in small numbers in the NM and, to a lesser extent, the CSA) were high in this FA, including cod *Gadus morhua* (22.77% of total FAs), halibut *Hippoglossus hippoglossus* (30.60%), mackerel *Scomber scombrus* (19.34%), pollack *Pollachius pollachius* (25.58%) and hake *Merluccius merluccius* (23.64%). Thus, in the present study a high amount of FA 22:6n-3 in *N. norvegicus* in the NM could indicate that the diet of *N. norvegicus* includes a significant amount of fish carcasses derived from discarded bycatches. Spawning grounds for cod and whiting are known to be present in the NM (Keltz & Bailey, 2010), whereas there is no spawning by these species around the Isle of Cumbrae area of the CSA (though some does appear to occur elsewhere in the area). However, in neither area is there any targeted commercial fishing for these species, although some commercially sized individuals of these species are sometimes landed, dependant on quotas.

20:1n-7

The FA 20:1n-7 was found in higher abundance in animals from the CSA (6.93%) compared with those from the NM (1.89%). Cook *et al.* (2010) showed that copepods were low in this FA and in addition the fish identified by Budge *et al.* (2002) as having large amounts of FA 22:6n-3 were low in FA 20:1n-7. This suggests that *N. norvegicus* in the CSA have a diet based more on sources other than fish, compared with *N. norvegicus* from the NM. However, there are no indications in the literature surveyed as to the possible identity of the source of this particular fatty acid.

22:0 NMID (non-methylene interrupted fatty acid)

The FA 22:0 NMID (non-methylene interrupted fatty acid) was also significantly higher in *N. norvegicus* from the NM, compared with those from the CSA, although the difference was not as great as for the two FAs mentioned above. Ackman & Hooper (1973) showed that this FA was present in *Littorina littorea* and other marine gastropod molluscs and it is known to be synthesised by marine invertebrates such as bivalve molluscs (Joseph, 1982). For this and other reasons, Budge *et al.* (2006) suggested that NMID FAs may be good indicators of

predation on bivalve molluscs. This suggests that the *N. norvegicus* from the NM may be consuming more molluscs than those from the CSA.

7.4.2 Month and sex interaction

Seasonal variation of FA signatures has been reported in the shore crab *Carcinus maenas* by Styrishave & Andersen (2000), who linked this to events in the life cycle of the crab such as moulting, reproduction or change in environmental condition. Since in *N. norvegicus* there was no significant clustering of sex, even when site and month were taken into account, in either the FA signatures or the average proportion within each FA family, this suggests that females are not operating with a feeding strategy different from males at any time of the year. This in turn suggests that variation in FA signatures among months is not explained by an effect of the reproductive cycle on feeding. Therefore FA signature differences throughout the year are probably based on the type of food available at the different times.

7.4.3 Comparisons with other studies

Tsape *et al.* (2010) analysed the FAs in the cephalothoraces of *N. norvegicus* from a Mediterranean population around Greece (no specific location given). Their methodologies differed in a number of ways from those of the present study, since they extracted the lipids from complete cepahalothoraces and did not identify either the sexes of the animals used, or the time of year when samples were obtained. For these reasons their results would reflect the contribution to the total FA amounts of tissues in the cephalothorax other than the hepatopancreas, for example the gonads, and in that case also by the state of maturation of ovaries in any females used. Also, these other tissues may have contained additional specific FAs not present in the hepatopancreas. In addition to these differences in sampling, their analytical methods were able to detect fewer FAs in total, so that any identified FA would have an apparently elevated percentage of the total, compared with the values calculated in the present study.

Nevertheless, taking all these factors into account, the results of Tsape *et al.* (2010) are consistent with the present findings from animals in the NM and CSA

in showing that FA 18:1n-9 was the most abundant FA. Thus, as a percentage of total FAs, the mean value of FA 18:1n-9 was 21.55% for the Greek population (from the complete cephalothorax), compared to 14.48% for the NM population and 13.51% for the CSA population (from the hepatopancreas). Falk-Petersen *et al.* (2000) indicated that 18:1n-9 is derived from animal-based prey items, which therefore suggests that *N. norvegicus* in the Greek Mediterranean area and the two Scottish fishing areas all have a high dependence on carnivory.

The FA 16:0 was also consistently high in *N. norvegicus* from these three areas, representing 19.29% of total cephalothoracic FAs in the Mediterranean (Tsape *et al.*, 2010), and 13.60% and 12.99% of the total hepatopancreatic FAs in the NM and CSA, respectively (present study). Rosa & Nunes (2002a) suggest that this FA is essential for reproduction.

The Greek study did not, however, find any 18:1n-7 (Tsape *et al.*, 2010), although it was found in all animals analysed in this present study (6.60% and 9.01% of total hepatopancreatic FAs for the NM and CSA, respectively). Indeed the lowest amount found in an individual from Scottish grounds was 3.18% in a female from the NM in December. Falk-Petersen *et al.* (2000) suggested that the presence of FA 18:1n-7 indicates herbivory as a potential feeding strategy, which would suggest that the animals from the West Coast of Scotland may have a greater proportion of plant-based items in their diet than *N. norvegicus* from Greek Mediterranean populations. These could be from drift algae, leaves of terrestrial plants washed into the sea by rivers, or even phytoplankton in the guts of copepods or bivalve molluscs.

7.4.4 Future work

The study reported in this chapter followed the first two approaches outlined by Budge *et al.* (2006) for examining the foraging ecology of a species, namely to identify the changes in FA signatures that occur through spatial and/or temporal variations in diet and to identify unique fatty acids found in a predator which can be traced to a single origin or prey species (see Section 1.1.4). However, it was beyond the scope of this investigation to follow the third approach (quantitatively estimating diet from FA signatures of predator and prey to determine the most likely trophic relationships).

As discussed above, analyses of the FAs which contributed most to the dissimilarity between sites, combined with reference to published work, allow some inferences to be made about possible trophic linkages. However, this has a number of limitations. Firstly, the published work was carried out in a number of different locations. Secondly, they were carried out at different times. Thirdly, the different studies identified different FAs, making direct comparisons difficult. All three limitations make it difficult to reach definitive conclusions about the trophic interactions of Scottish *N. norvegicus* populations.

It may be fruitful in future studies aimed at determining the trophic dynamics of *N. norvegicus* to take an ecosystem approach, as described, for example, by Spilmont *et al.* (2009), which examines how the food web is constructed. This type of study may also benefit from being linked to stable isotope analysis, determining, for example, the values of δ^{15}N and δ^{13}C of the possible prey items identified from the FA analysis described in this chapter. The developing field of compound-specific stable isotope analysis could also be used, to determine, more definitively, the source of the FAs by comparing the δ^{13}C values of FAs between *N. norvegicus* and its possible prey. Evershed *et al.* (2007) reviewed this procedure and it has been utilised in a number of studies, such as that of Pond *et al.* (2008).

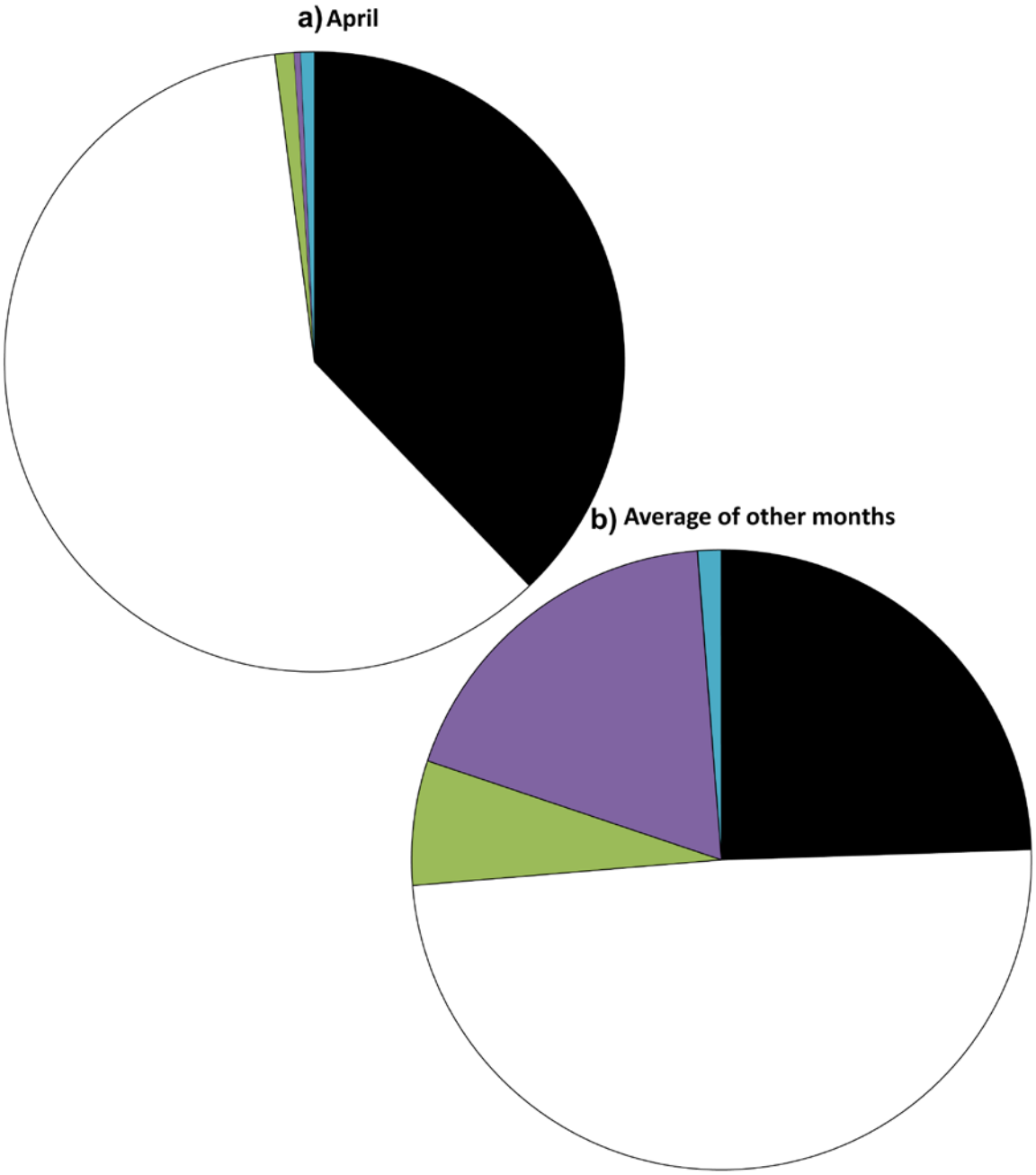

Figure 7.2 Pie charts of fatty acid family distribution in a) samples from the Clyde Sea Area in April which were subject to oxidation and b) the rest of animals selected from the Clyde as an average. Note the reduction in Polyunsaturated Fatty Acid (PUFA) 3 and 6 in the oxidised samples. Black- saturated Fatty Acid (SFA), White- Monounsaturated Fatty Acids (MUFA), Green- PUFA 3, purple PUFA 6, Blue- other FAs. a) n=6, b) n=31

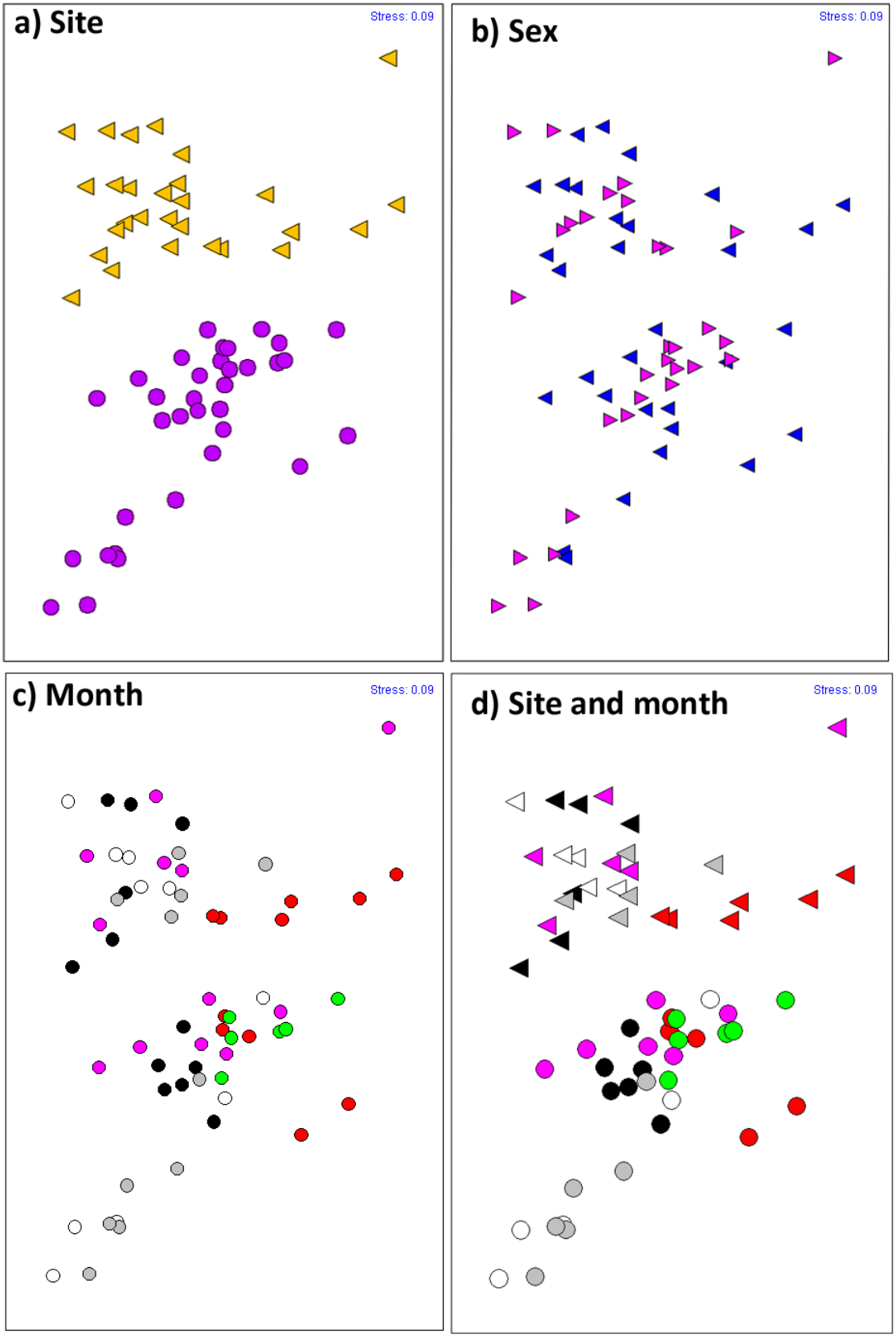

Figure 7.3 Multi dimensional scaling plots of all the fatty acid signature of each *N. norvegicus* analysed. Animal samples grouped together to note effect of explanatory factors of a) Site [Purple circles - North Minch, Orange triangles - Clyde Sea Area], b) Sex [Blue-Male, Pink-Female], c) Month [Feb - Red, Apr - Green, Jun - Pink, Aug - Black, Oct - White, Dec - Grey] and d) Site and Month interaction [shapes as a)], [colours as c)]. N=62

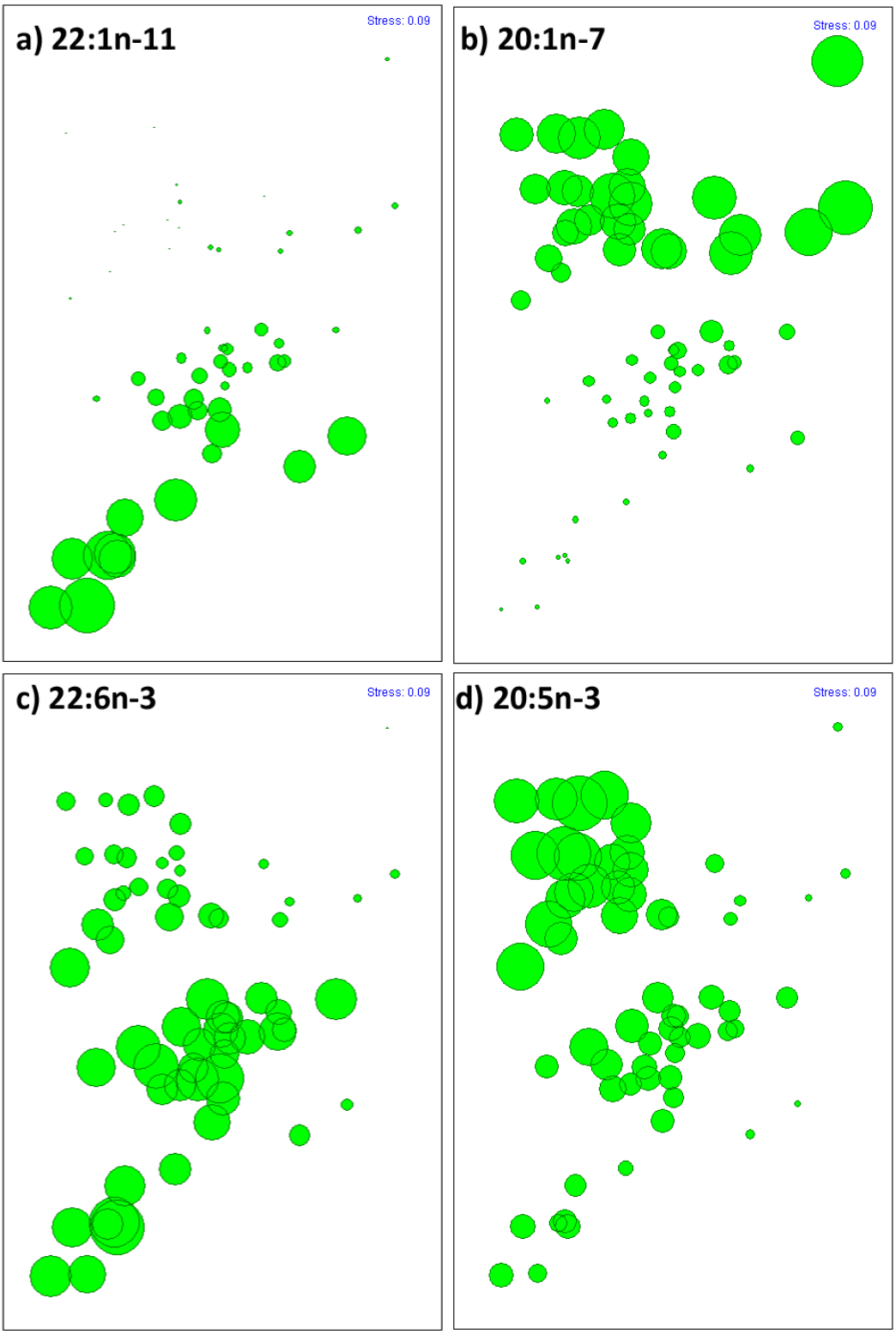

Figure 7.4 Multi-dimensional scaling plots of all the fatty acid signature of each *N. norvegicus* analysed. Animal samples shown with the relative abundance of a) 22:1n-11 b) 20:1n7 c) 22:6n-3 d) 20:5n3 fatty acids. Diameter of the circle is in proportion to the amount of that fatty acid present. n=62

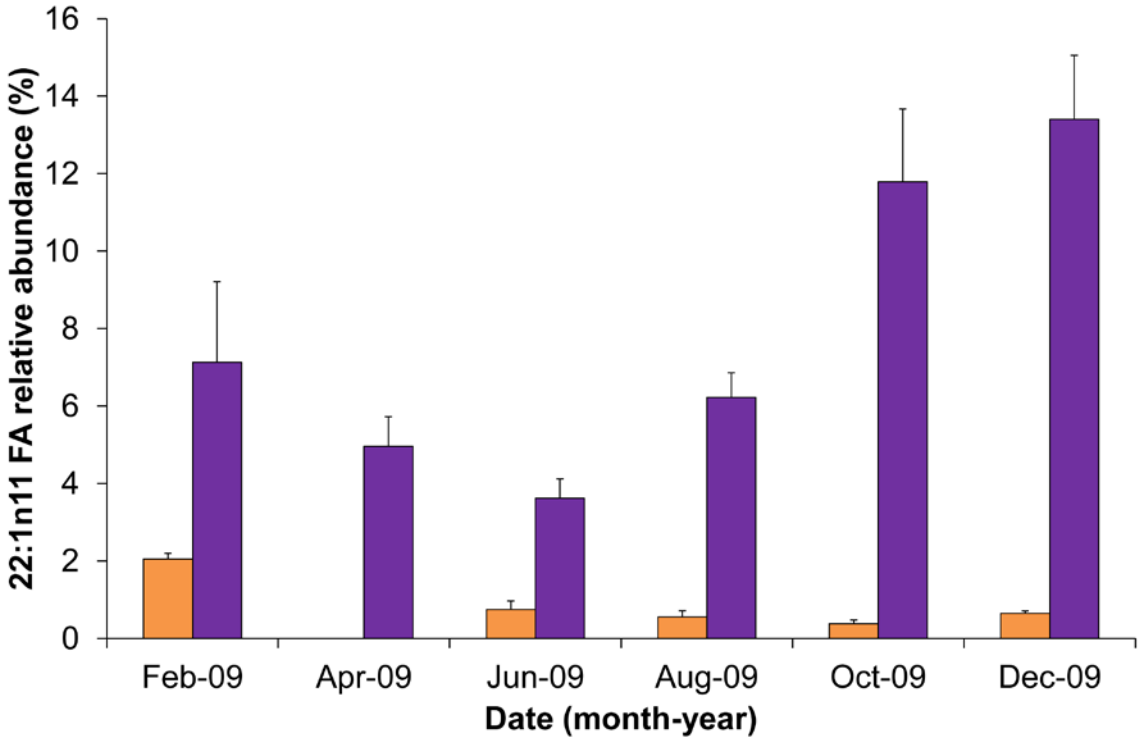

Figure 7.5 The abundance of FA 22:1n-11 as a percentage of total FAs in the hepatopancreas tissues of *N. norvegicus* from the CSA (orange bars) and NM (purple bars). Error bars represent one standard error. n=62

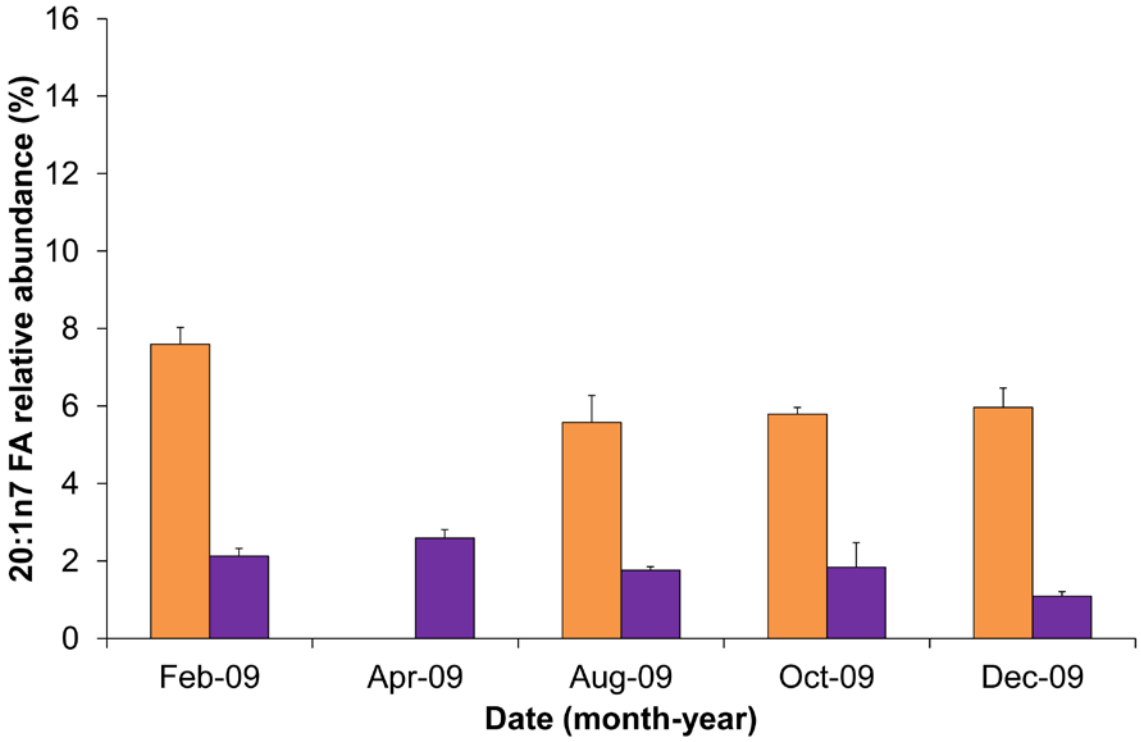

Figure 7.6 The abundance of FA 20:1n-7 as a percentage of total FAs in the hepatopancreas tissues of *N. norvegicus* from the CSA (orange bars) and NM (purple bars). Error bars represent one standard error. n=62

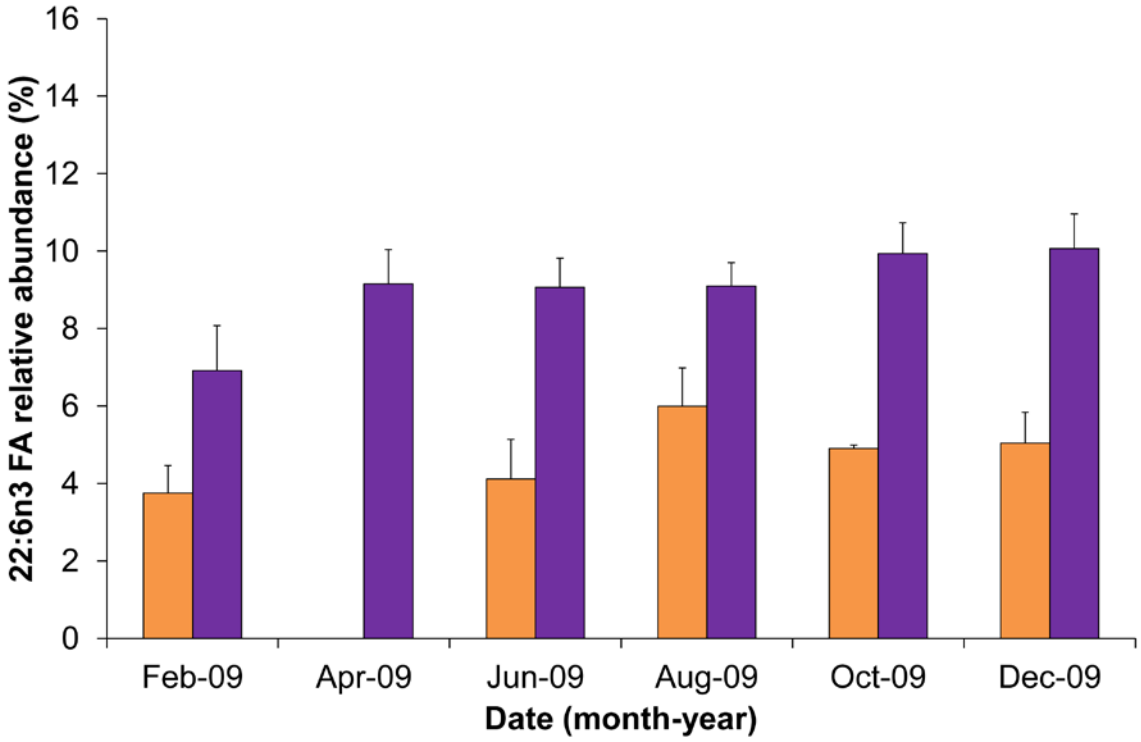

Figure 7.7 The abundance of FA 22:6n-3 as a percentage of total FAs in the hepatopancreas tissues of *N. norvegicus* from the CSA (orange bars) and NM (purple bars). Error bars represent one standard error. n=62

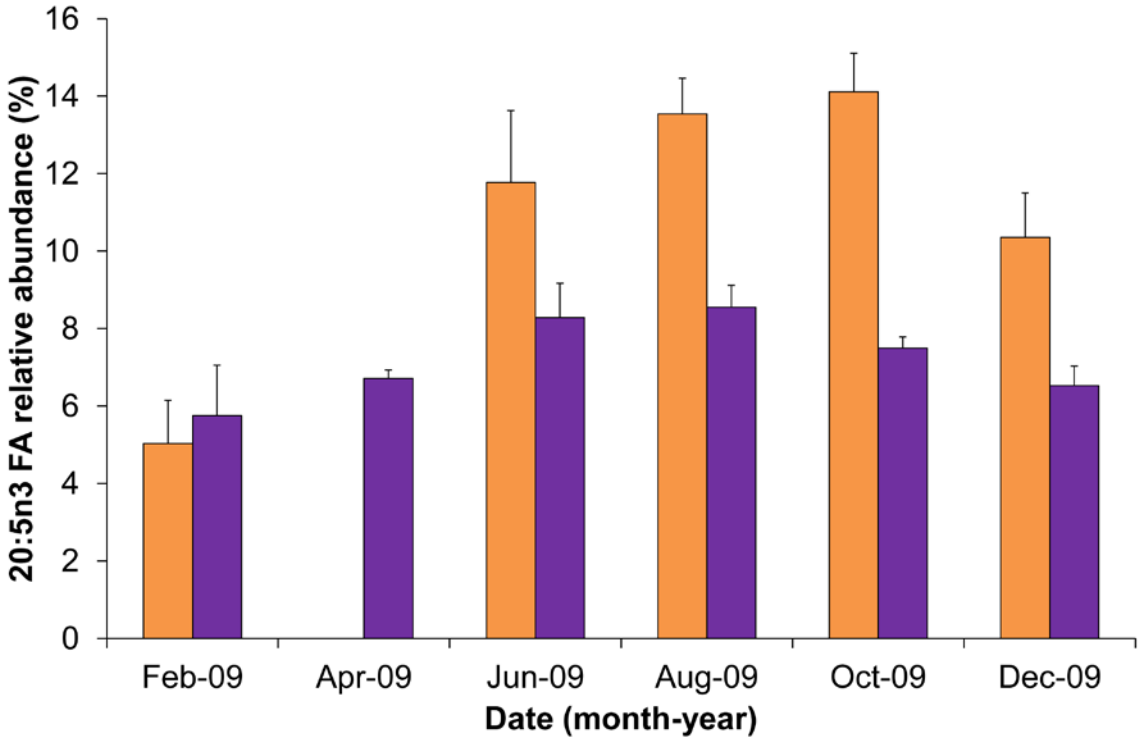

Figure 7.8 The abundance of FA 20:5n-3 as a percentage of total FAs in the hepatopancreas tissues of *N. norvegicus* from the CSA (orange bars) and NM (purple bars). Error bars represent one standard error.

Table 7.10 List of fatty acids identified, with names when available.

	Systematic name	**Trivial or other name**
14:0	tetradecanoic	Myristic acid
anteiso 15:0		
iso 15:0		
15:0	pentadecanoic	
iso 16:0		
16:0	hexadecanoic	Palmitic acid
anteiso 17:0		
iso 17:0		
iso 18:0		
18:0	octadecanoic	Stearic acid
anteiso 19:0		
iso 19:0		
19:0	nonadecanoic	
20:0	eicosanoic	Arachidic acid
22:0	docosanoic	Behenic acid
24:0	tetracosanoic	Lignoceric aicd
16:1n-9	*cis*-9-hexadecenoic	Palmitoleic acid
16:1n-7	*cis*-7-hexadecenoic	Myristoleic acid
17:1		
18:1n-9	*cis*-9-octadecenoic	Oleic acid
18:1n-7	*cis*-11-octadecenoic	*cis*-Vaccenic acid
19:1		
20:1n-11		
20:1n-9	*cis*-11-eicosenoic	Gondoic acid
20:1n-7		
22:1n-11		Cetoleic acid
22:1n-9	*cis*-13-docosenoic	Erucic acid
22:1n-7		
24:1n-9	*cis*-15-tetracosenoic	Nervonic acid
18:2n-6	*all-cis*-9,12-octadecadienoic acid	Linoleic acid (LA)
18:3n-6	*all-cis*-6,9,12-octadecatrienoic acid	Y-Linolenic acid (GLA)
20:2n-6	*all-cis*-11,14-eicosadienoic acid	Eicosadienoic acid
20:3n-6	*all-cis*-8,11,14-eicosatrienoic acid	Dihomo-gamma-linolenic acid (DGLA)
20:4n-6	*all-cis*-5,8,11,14-eicosatetraenoic acid	Arachidonic acid (AA)
22:4n-6	*all-cis*-7,10,13,16-docosatetraenoic acid	Adrenic acid
22:5n-6	*all-cis*-4,7,10,13,16-docosapentaenoic acid	Docosapentaenoic acid
18:3n-3	*all-cis*-9,12,15-octadecatrienoic acid	α-Linolenic acid (ALA)
18:4n-3	*all-cis*-6,9,12,15-octadecatetraenoic acid	Stearidonic acid (SDA)
20:3n-3	*all-cis*-11,14,17-eicosatrienoic acid	Eicosatrienoic acid (ETE)
20:4n-3	*all-cis*-8,11,14,17-eicosatetraenoic acid	Eicosatetraenoic acid (ETA)
20:5n-3	*all-cis*-5,8,11,14,17-eicosapentaenoic acid	Eicosapentaenoic (EPA)
22:5n-3	*all-cis*-7,10,13,16,19-docosapentaenoic acid	Docosapentaenoic acid (DPA), Clupanodonic acid
22:6n-3	*all-cis*-4,7,10,13,16,19-docosahexaenoic acid	Docosahexaenoic acid (DHA)
16:2		
16:3		
16:4		
16:0 DMA		
18:0 DMA		
18:1 DMA		
22:0 NMID		

8 General discussion

The overall aim of this study was to define aspects of *Nephrops norvegicus* nutrition in animals caught off the west coast of Scotland. This study looked at how biotic and abiotic factors influence the nutrition of this species. To do this, two aspects of nutrition were considered: firstly, a study of the nutritional status of *N. norvegicus*, compiling a profile of their overall physiological reactions to starvation (Chapters 2-5); and secondly, an investigation of the trophic dynamics of *N. norvegicus*, indicating the types of food consumed: the trophic position and the identities of the dietary items consumed (Chapters 6-7).

8.1 Nutritional status

McCue (2010) stated that the starvation responses of animals can involve two phases. First there is the metabolic depression phase, where the metabolic rate is reduced thereby reducing the energy requirements, and thus in turn the use of resources. Secondly, there is the utilisation of metabolic reserves for energy acquisition. The first half of this thesis (Chapters 2-4) dealt with the nutritional status of *N. norvegicus* in a series of controlled starvation trials, in an attempt to identify the timing and extent of the two phases of its starvation response, *sensu* McCue (2010). Chapter 2 dealt with the effect of starvation on male *N. norvegicus* over a 20 week period. Chapter 3 then considered the effect of starvation on females over the same period, along with a further study of males. Using the results of these studies, in Chapter 4 an attempt was made to create a biomarker model for indicating the extent of the starvation responses of field-caught animals. Chapter 5 then applied this model to measurements obtained from *N. norvegicus* from two study sites, namely the Clyde Sea Area (CSA) and the North Minch (NM), thus providing an indication of whether they were expressing starvation responses at either site or in any season.

8.2 Aquarium starvation studies

Previous studies on the effects of starvation on *N. norvegicus*, such as those of Dall (1981), Baden *et al.* (1994), Parslow-Williams *et al.* (2002) and Mente *et*

al.(2011), all noted the biometric and biochemical changes which occur over time. The main findings of these studies are summarised in Table 2.3.

In the present study, there was a clear and significant reaction to starvation in both male and female *N. norvegicus*, with five of the parameters measured showing a clear difference over the 20 week starvation period in males, and four in females. In both males and females the copper concentration of the hepatopancreas, the lipid and water content of the hepatopancreas and the HSI ratio changed significantly during starvation. The C:N ratio of the tail muscle fell significantly only in males.

Dall (1981) concluded that a metabolic reduction (depression) strategy enabled *N. norvegicus* to avoid calling on lipid reserves during a starvation period of 18 days, consistent with other authors (such as Zhang *et al.*, 2009) who had shown that decapods utilised lipids only after a period of many days. The present study, however, considered time scales substantially greater than 18 days. If, in the field, females were deprived of access to food when restricted to their burrows over the period of egg brooding, then a starvation period of up to 6 months would occur (even longer in more northerly areas). Therefore the trials reported in Chapters 2 and 3 were carried out for around this period of time (i.e for 20 weeks with intermediate sample points at every 4 weeks). The results obtained in this study conform with Dall's (1981) findings, in that the paired measures of copper concentration in the haemolymph and in the hepatopancreas suggest that a strategy of metabolic depression alone is followed by males for around 12 weeks and by females for around 20 weeks, prior to lipids being catabolised within the hepatopancreas. Unlike Dall (1981), the present study identified a time point beyond 18 days when lipid reserves were called upon, although Dall (1981) may never have considered that 12 weeks was an ecologically relevant time scale to consider. However, the finding that these animals can survive beyond 20 weeks of food deprivation using these mechanisms is a significant one.

It was difficult to interpret measures of carbohydrates in *N. norvegicus* in terms of their reaction to food deprivation, due to the stress effects imposed by the trawling method used to obtain the animals, as described by Albalat *et al.* (2009). Essentially, carbohydrate stores are severely depleted by exertional

exhaustion, as the animals react to the fishing gear and attempt to escape from the net. In the subsequent starvation trials the carbohydrate in the hepatopancreas and tail muscle did recover in the control group that was fed, although this took longer than the pre-trial period of two weeks that had been allowed. In the experimental group which was food-deprived, however, these carbohydrate reserves never recovered from their low post-capture levels. It thus seems that no net production of carbohydrate is possible under conditions of food deprivation.

Proteins in the tail muscle did not decline significantly during the 20 week period of the starvation trial. This is consistent with the studies by Parslow-Williams (1998) and Mente *et al.* (2011) which both showed that protein did not decrease in the tail muscle over these time scales. As pointed out by Taylor & Anstiss (1999), however, the haemocyanin decrease which occurs as a response to starvation may reflect not only an onset of metabolic depression, but could also provide a protein resource that could contribute to the production of energy over the first 12 week period of starvation. A study of the metabolic handling of the haemocyanin under starvation conditions (including the extent of excretion of nitrogenous breakdown products) could establish if this was the case.

In the present study, the hepatopancreas appeared to be the major source of utilised reserves as a response to starvation, whereas the tail muscle was seemingly utilised very little. Confirmation of this difference was provided by measures of the water content of the tissues, since catabolised resources such as lipids in the tissue are then replaced by water. The hepatopancreas water content increased by around 12% in males and was significantly higher in individuals sampled at week 12 than in animals sampled at week 0. On the other hand, increases in the water content of the tail muscle were not significant. This difference is possibly due to the fact that the hepatopancreas contains a lot more lipids than the tail muscle, much of which is required for moult-related processes. H.Philip (pers. comm.) has found moulting to be suppressed in food-deprived *N. norvegicus* under experimental conditions, as was also seen in this study. It might therefore be predicted that the utilisation of essential lipids for energy, as in phase 2 of the starvation response, might disrupt the moulting process.

In comparison, the lipids in the tail represent as little as *ca* 1% of the tail muscle mass. These lipids, in the tail are probably structural cell membrane phospholipids, rather than a reservoir of lipid energy reserves. Results in Chapter 2 suggest that they may have undergone a significant reduction, indicative of these reserves in the tail being utilised during the starvation period, although measuring such low amounts of lipids can be imprecise. In a practical sense, because the tail muscle has such small lipid reserves it may not be appropriate to use changes in these as a biomarker of starvation in field-caught samples.

However, it was found in the first trial with male *N. norvegicus* that the C:N ratio in the tail muscle did decrease significantly with greater starvation (Figure 2.11), and that there was a clear relationship between this and the lipid content of this tissue, consistent with the results of Haubert *et al.* (2005). The C:N ratio may therefore provide a better monitor of resource utilisation from the tail muscle, but even for this, the effects were not statistically significant in all trials.

In the study of females, the carapace from severely food-deprived *N. norvegicus* (24 weeks) was analysed for the isotope ratio δ^{15}N. This showed it was significantly lower than that in females taken directly from the sea in the CSA. The HSI measures in these animals confirmed that the field samples were well-fed in comparison to the severely food-deprived group. This initial finding suggests that the carapace, which is often regarded as an inert material, could in fact be an additional source of metabolisable reserves, which could contribute to the nutritional status of the individual, it is well known that crustaceans deposit and recover calcium, protein and other materials to and from the exoskeleton during the moult cycle (Chang, 1995), so mechanisms do exist for mobilising reserves from the carapace. The consequences of this are to fully understand how *N. norvegicus* responds to starvation: all parts of the body (tail, hepatopancreas, gills, carapace etc.) need to be measured to look for responses to starvation. A targeted study is required to determine this more systematically.

The effect of temperature on the response to food deprivation in *N. norvegicus* was also considered. In the second starvation trial both males and females were held at a lower temperature (9.4 ± 0.6°C), compared with the one used in the

first trial on males (11.7 ± 0.1˚C). This showed that the threshold of the starvation response is temperature-dependent, for males being in week 12 at 11.7˚C, but in week 20 at 9.4˚C (Chapter 4). It was found that both sexes seemed to react similarly in the second trial (at 9.4˚C), in terms of copper increasing and lipids decreasing in the hepatopancreas, it is concluded that the responses of the two sexes to starvation are essentially the same. However the ST was calculated from the first male trial, because the 2nd male trial (same temperature as the female trial) was conducted with small numbers of animals so would not be statistically rigorous.

Such results are perhaps not surprising since the powerful effect of temperature on the metabolic rate of animals is well known. However, they do emphasise the importance of a strict regulation of temperature, in starvation studies; and also ensuring that comparisons are made at the same temperature. Perhaps also a range of temperatures should be used in order to establish the temperature-dependence of potential biomarkers.

The copper concentration of the hepatopancreas in this study has been used as a proxy measure for metabolic rate, on the basis that Parslow-Williams (1998) showed a reduction in metabolic rate in *N. norvegicus* measured via O_2 consumption after 8 weeks of starvation. The concentration of the O_2 carrying protein haemocyanin has also been shown to decrease as a result of this metabolic depression (Depledge & Bjerregard, 1989). This means that there is a concomitant reduction of copper that is associated with the haemocyanin in the haemolymph, as shown by Spoek (1974) and Baden *et al.* (1990). Taylor & Anstiss (1999) suggest that the fate of this copper is to be translocated to the hepatopancreas where it is stored. In this study, the depletion of copper in the haemolymph and the increase of copper in the hepatopancreas have been found to occur over the same time scale as the metabolic rate decrease measured by Parslow-Williams (1998). The validity of using the copper concentration in the hepatopancreas as a proxy measure for metabolic depression is based upon this sequence of evidence, as summarised in Figure 8.1. It would be helpful now to assess the reliability of using hepatopancreas copper concentration as a proxy measure for metabolic rate more rigorously.

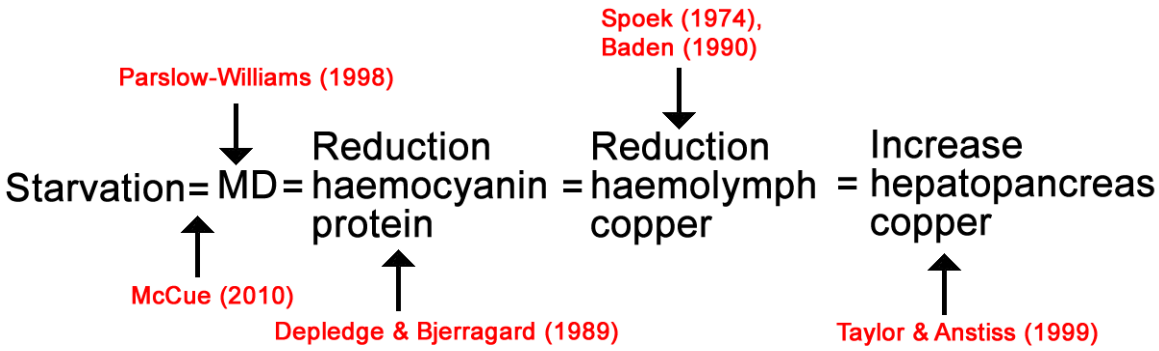

Figure 8.1 Evidential basis for using the increase in the concentration of copper in the hepatopancreas as a proxy measure of metabolic depression (MD) in *N. norvegicus* and other crustacean species, based on a number of different studies.

In the present study the detection limits (the critical threshold point- CTP in Chapter 4) were defined as 12 weeks starvation for males held at 11.7°C, and 20 weeks for males and females held at 9.4°C. It is assumed that before the CTP the *N. norvegicus* are nevertheless reacting to starvation in ways that are at that time not detectable. Further work using more sensitive and specific measurements could reduce these detection limits. Also, if a larger sample size of animals was used with the present measurements the CTP may have been detected earlier, depending on the time course of response of the particular parameter. A logistic-type curve could also be a flexible way of representing the range of these different possible responses.

A shortcoming of this and many other starvation studies quoted is that there is an 'all-or-nothing' approach to both the imposition of starvation and the interpretation of the responses. The fed group is often provided with food items *ad lib.,* whereas the unfed group has complete withdrawal of food for the whole period of starvation. This is unlikely to be ecologically realistic for *N. norvegicus* (or indeed any other animal) in terms of either the extent of food withdrawal or its continuous exclusion.

The findings made in this study have implications outwith the field of *N. norvegicus* biology. For example, the holding and impounding of the larger *Homarus gammarus* lobsters by Scottish fishermen is a growing business.

Companies buy up lobsters in the late summer, when the price is low, and then impound them for several months, so that they can attract much higher prices on the Christmas market (D.M.Neil pers. comm.). The holding arrangements used by these companies involve keeping the lobsters either communally or separated in baskets, but in either case they are provided with food items such as fish offal. However, this can lead to fouling of the tanks from unconsumed food, which also encourages the multiplication of spoilage and other bacteria. Outbreaks of bacterial diseases such as Gaffkemia (due to the Gram-positive bacterium *Aerococcus viridans* var *homari*) have in fact been reported, and have led to the mass mortality of complete impoundments of lobsters, with consequent loss of earnings.

The present study suggests a better strategy for impoundment of lobsters, whereby they are not fed at all (thus avoiding potential fouling of the water), but rather the holding temperature of the water is reduced to more certainly induce metabolic depression. It is predicted from the results obtained on *N. norvegicus* that although there may be a reduction of resources in the hepatopancreas of *H. gammarus,* the tail muscle would probably not be affected by this type of impoundment. This certainly seems to be the case in the Canadian industry for the American lobster, *H. americanus*. These lobsters are successfully held for several months without feeding in impoundments with water temperatures of *ca* 5°C, without any apparent detriment to flesh quality Garland & Uglow (2011).

8.3 Field based observations

The second half of the thesis (Chapters 6-7) was concerned with the trophic dynamics of *N. norvegicus* at two sites off the west coast of Scotland. Here the aim was to expand the knowledge on the types of food consumed by *N. norvegicus*. This information, along with nutritional status measurements in Chapter 5, was considered in relation to season, sex and site to answer the following questions.

- With regard to season, is there a reduction in nutritional condition of *N. norvegicus* over the winter due to a decrease in primary production?

- With regard to sex, do females have a reduced nutritional status compared with males when they emerge from their winter brooding period, indicating that they undergo a period of starvation over the winter? Also, do females employ a filter feeding strategy over the winter months, as suggested by Parslow-Williams (1998)?

- With regard to site, do *N. norvegicus* in the CSA feed differently from *N. norvegicus* in the NM, and can this be seen in fractional trophic level differences and differences in fatty acid signatures?

8.3.1 Season

The winter would be the most obvious time for *N. norvegicus* to experience degrees of starvation in the wild, for although bottom water temperatures may be little different from those in the summer (Albalat *et al.*, 2010) and the effect of temperature would be unlikely to influence the amount of food consumed, nevertheless the numbers of pelagic phytoplankton are low and therefore the energy entering the benthos is reduced. This is probably why *N. norvegicus* has evolved responses to survive for so long when food is scarce. In 2009, the year that was monitored, the nutritional status of both males and females was low at the beginning of the year in both the CSA and the NM. This suggests that food might have been scarce over the winter of 2008 – 2009. However, the nutritional status of both males and females increased over the year, so that by the early winter of 2009 - 2010 it was higher than at the beginning of the year. This is indicative of an increase in food consumption, and suggests that either: food limitation over the winter may not be a feature that occurs every year; or this study has measured the end of the winter (2008/2009) when food has not been available and measured the beginning of the winter in (2009/2010) which is at the end of the main 'feeding season' (autumn/early winter) and if this study measured into 2010 a drop could have been seen.

8.3.2 Sex

Differences between the sexes were measured primarily to determine if the nutrition of female *N. norvegicus* changed due to them residing within their burrows over the winter period.

No evidence was obtained to suggest that females were in a state of advanced starvation over the winter due to the brooding behaviour they exhibit. There were some indications of partial starvation through the winter, but these were also recorded for males; it may have been due to reduced food availability in the winter. However, in the months of May - August females had significantly higher concentrations of copper in their hepatopancreas than males, though below the threshold of starvation (indicated by laboratory studies), and thus indicative of a slightly reduced metabolism compared with males over these same months. This could be due to them being in a more sedentary state during this time, compared with males. However as the sex ratio values of these months indicate that females have emerged from burrows at this point, this is unlikely. If this is found to occur each year then this would need to be studied further.

Parslow-Willams (1998) suggested that females could use 'filter' feeding (as shown by Loo *et al.* (1993)) as a strategy to feed over their winter brooding period, and suggested that this could be tested directly using the value of $\delta^{15}N$ in these animals. This was done in the present study for both males and females throughout the year. If females did use a filter feeding strategy then they would have a significantly less positive $\delta^{15}N$ at the end of the brooding period compared with feeding on 'normal' scavenged and predated food items throughout the rest of the summer. This, however, was found not to be the case as there was no significant variation in $\delta^{15}N$ between male and female *N. norvegicus* at either site at the end of the brooding period. Neither was there any significant difference between the fatty acid signatures of male and female *N. norvegicus* at either site (Chapter 7).

Therefore all evidence in this study suggests that females are not starving over the winter as a result of brooding their eggs, although there may have been a reduced amount of feeding. The trophic level (shown by stable Isotopes) and the type of prey consumed (shown by fatty acid analysis) are not statistically different between the two sexes.

8.3.3 Site

The final factor considered in this study was a site-related difference in the nutritional status of *N. norvegicus* from the CSA and the NM. Both Tuck *et al.* (1997c) and Parslow-Williams *et al.* (2001) suggested that density-dependent factors influence the nutritional status of *N. norvegicus* in different areas of the CSA. Tuck *et al.* (1997c) also showed that their growth was negatively correlated with burrow density, and surmised that this is due to the fact that at higher densities less food is available to individual animals through greater competition. As the burrow density in 2009 in the CSA (0.85 m^{-2}) was higher than that in the NM (0.55 m^{-2}) it is hypothesised that the nutritional status of *N. norvegicus* from the CSA will be lower than that of the NM. The findings in Chapter 5 showed that the water and the copper concentration in the hepatopancreas of *N. norvegicus* in the CSA are higher than in *N. norvegicus* from the NM, suggesting that the *N. norvegicus* in the CSA area have a lower nutritional status than *N. norvegicus* in the NM. This is likely to be due to the density-dependent factors discussed.

There was also a clear and highly significant difference in *N. norvegicus* sampled from the CSA and NM in terms of $\delta^{15}N$ and $\delta^{13}C$ values (Chapter 6). The correct interpretation of these results required a trophic baseline to be established, to remove any site-specific confounding factors. For this purpose, the scallop *Aequipecten opercularis* was used as a trophic base, which was prescribed by Jennings & Warr (2003) as being more reliable than using particulate organic matter (POM). When the trophic baseline was applied to the trophic level equation (as displayed in Chapter 6, Equation 6.3), *N. norvegicus* from the NM were found to be feeding at 0.6 trophic-level units higher than those in the CSA.

The $\delta^{15}N$ of *A. opercularis* was also higher in the CSA than in the NM, suggesting that there was site enrichment from another nitrogenous source. As stated in Chapter 6, the CSA receives around 60 kt y^{-1} of nitrate in comparison to the NM, which receives only <10 kt y^{-1} input (Baxter *et al.,* 2008) which is likely to be one of the main reasons for the increased baseline of $\delta^{15}N$ in the CSA.

Alongside the collection of *A. opercularis* and *N. norvegicus,* four other species were sampled at both sites within a period of one week. Individuals of all these species from the CSA had an elevated δ^{15}N compared with individuals of the same species from the NM. A general conclusion that can be drawn from this that these species could be used as sentinels to measure the state of these marine areas in terms of nitrogenous impact. For this purpose, *N. norvegicus* is probably not the best choice as a sentinel species for anthropogenic impacts, since the difference in its δ^{15}N between the two sites was less than for the other species.

Another important conclusion from this stable isotope analysis arises from the fact that the δ^{13}C for the *N. norvegicus* from the CSA was less negative than for the same species from the NM. This indicates that the food web in the CSA is based more around a benthic food supply, whereas in the NM it is more a pelagic-driven food supply, as described by Boutton (1991); Michener & Kaufman (2006) and Barnes *et al.* (2009).

A site difference between *N. norvegicus* in the CSA and the NM is also supported by finding a clear and significant difference in the fatty acid (FA) signatures. The FA 22:1n-11 was more abundant in the NM animals than in those in the CSA. This FA is derived from pelagic sources and is in high concentrations in planktivores such as mackerel and herring. The FA 18:1n-7, which is indicative of herbivorous feeding, was present in animals from both sites. To what extent this type of feeding is employed by *N. norvegicus* is not known, but this finding suggests that this should be examined in the future. Both sites were also high in the FA 22:6n-3 (docosahexaenoic acid), which is indicative of demersal fish such as cod (*Gadus morhua*), haddock (*Melanogrammus aeglefinus*), and hake (*Merluccius merluccius*). This suggests that the flesh of fish could be an important food supply for *N. norvegicus*. A contribution of fish to the diet of *N. norvegicus* may therefore have been underestimated in studies such as those of Thomas & Davidson (1962) and Parslow-Williams *et al.* (2002), which used gut content analysis (GCA) and came to the conclusion that animals with harder structures were the major part of their diet. By its nature, this method may severely underestimate the amount of fish consumed, unless the otolith or larger bones are present.

It is difficult to imagine that *N. norvegicus* would be able to capture fish that are swimming near the sea bed; it is much more likely that they might scavenge the carcasses of dead fish which fall from above. A number of lines of evidence support this suggestion. Ramsay *et al.* (1997) showed that food-falls from fisheries discards provide energy subsidies to the benthic scavengers, and may provide up to 11% of the energy requirements of benthic scavengers. Kaiser & Hiddin (2007) reviewed various studies that show that benthic scavenging also occurs in the benthos of continental shelf fisheries, and suggested that this energy subsidy to marine food webs would otherwise not be available to these benthic scavengers. Oakley (1978) showed that *N. norvegicus* are likely to scavenge on dead and decaying carrion that enter the benthos.

The CSA and the NM are areas that are subjected to heavy fishing pressure, primarily from trawlers that target *N. norvegicus* itself. For example, there was 3,497 tonnes of *N. norvegicus* landed in the NM in 2009 (ICES, 2011), and the trawls catches comprised on average 56% of the target species and 44% of bycatch, with virtually all of the latter being discarded (Milligan & Neil, in prep). The discarded bycatch included round fish, flat fish, elasmobranchs and invertebrates; the roundfish comprised both commercial species such as cod, haddock and whiting, and non-commercial species such as pout (*Trisopterus luscus*) and ling (*Molva molva*). Therefore, a large quantity of fish is being discarded from these fishing vessels, much of which will reach the benthos and some of it may be consumed by *N. norvegicus* there.

Working with these values of bycatch it is possible to calculate how many tonnes of material are being discarded annually from trawlers in the NM. Of the initially discarded material from the vessels, not all of it will reach the benthos; for instance, a proportion of it will be removed by scavenging sea birds. Garthe *et al.* (1996) surveyed the amount of discards that is scavenged by sea birds in the North Sea mixed fishery, in relation to the amount in the initial catch. Wieczorek (1999) showed that no significant scavenging of discards occurs as it falls through the water column. Applying these figures to the NM fishery allows the percentage of discards that enters the sea to be estimated (i.e. amount initially discarded minus amount scavenged by sea birds). Controlling for the area of the NM and burrow density it is possible to calculate the amount of discarded material per burrow (Table 8.1).

This indicates that 0.264 $g.m^{-2}.y^{-1}$ of discards entered the benthos in the NM in 2009. As the burrow density of the NM in 2009 was 0.55 m^{-2}, this yields a value of 1.31 x 10^{-3} g of discards per burrow per day. Mente (2010) indicated that the daily food intake of an individual *N. norvegicus* was around 0.6 $g.d^{-1}$(under aquarium conditions at 11 ± 1˚C with a 12:12h light:dark photoperiod). If it is assumed that there is one *N. norvegicus* in each burrow (probably an underestimate), then the amount available per individual would represent about 0.22% of its daily food intake.

Table 8.Estimation of the amount of discards from *N. norvegicus* fishing vessels in the NM in 2009, which could be available as food for *N. norvegicus*. C- amount caught by fishermen, B-amount scavenged by birds, p-percentage, t- tonnage,). Bold values are reported values, normal numbers are calculated.

	Total caught 2009		Scavenged by sea birds		discards entering sea
	Cp[1] (%)	Ct (tonnes)	Bp[3] (%)	Bt (tonnes)	Dw (tonnes)
N. norvegicus landed	**56**	**3,497**[2]	-	-	-
N. norvegicus discarded	**6**	375	**88**	328	47
Round fish	**17**	1,062	**79**	834	227
Flat fish	**3**	187	**13**	24	164
Elasmobranchs	**12**	749	**13**	100	649
Invertebrates	**6**	375	**6**	23	352
	100	6,245		1,308	**1,439**
				Area km^2	5,426
				$g.m^2.y^{-1}$	0.264
				$g.burrow.d^{-1}$	1.31 x 10^{-3}

[1]Milligan & Neil (in prep)
[2]ICES (2011)
[3]Garthe *et al.* (1996)

Of course this can only be regarded as an approximation, and is subject to numerous limitations. For example it assumes an even distribution of discarded material over the whole NM area; it ignores the effects of tides and currents which might carry material away; it omits the effect of scavenging species other than *N. norvegicus* in the benthos as well as the survival of certain species discarded, and thus in reality could be much lower than estimated.

Therefore this suggests that fisheries bycatch is not a large subsidy to the diet of *N. norvegicus*, and the interpretation that fish make up a substantial proportion of diet of *N. norvegicus* in the NM may not be valid. As discussed in Chapter 7 the FA of 22:1n-11 and 22:6n-3 could in fact have come directly from dead

copepods entering the benthic food chain, and thus be higher in amount in the NM than the CSA, due to depth. This is also supported by the fact that $\delta^{13}C$ values of all species measured in the NM in this present study indicate pelagic based food webs. To be sure about the origins of these fatty acids, a compound specific stable isotope analysis would be beneficial. Here the $\delta^{15}N$ of the specific fatty acids (22:1n-11 and 22:6n-3) would indicate if they are from a fish based diet (as $\delta^{15}N$ would be in the range of the bulk fish $\delta^{15}N$) or a copepod based diet (in that case $\delta^{15}N$ would be lower than the $\delta^{15}N$ for fish and more in the range of bulk copepod $\delta^{15}N$). If this method did indeed indecate that *N. norvegicus* are consuming fish then all potential sources of input, including the natural mortality of fish in addition to the discarded bycatch from fisheries, would need to be considered.

8.4 Conclusions

To conclude, this study has reinforced the concept set out by McLeod *et al.* (2004) and Sanchez-Paz *et al.* (2006) that the physiological condition and changes to the condition in decapod crustaceans are very rarely universal across species, and that species-specific studies are required to determine the particular responses of *N. norvegicus* to starvation. Also, the effect of external factors such as temperature, depth, trophic baseline values, nitrate levels in the sea, as well as density-dependent factors and fisheries impacts, all need to be considered to fully understand the feeding behaviour and trophic dynamics of this species.

A larger study utilising *N. norvegicus* from across its full geographic range (Figure 1.5) will make it possible to determine whether the differences between the two sites found in the present study in terms of trophic levels and diets are typical of the variations found more widely.

Finally, more work clearly needs to be carried out on the effect of discarding on the feeding habits, and thus the population dynamics, of *N. norvegicus,* to ensure the continuation of sustainable *N. norvegicus* fisheries.

Appendices

Table A.1 Number of observations of male *N. norvegicus* in each unfed and unfed group at each week point for each parameter measured, as used in Chapter 2.

initial n=61	unfed						fed						
Measurement	**0**	**4**	**8**	**12**	**16**	**20**	**0**	**4**	**8**	**12**	**16**	**20**	**n**
Carapace length (mm)	4	4	5	6	5	4	3	6	6	6	6	5	60
Hepatopancreas copper (µg.g^{-1} wet)	4	5	5	4	4	2	3	6	6	5	6	5	55
Haemolymph copper (µg.g^{-1} wet)	4	4	5	6	5	2	3	6	5	6	6	3	55
Hepatopancreas glycogen (mg.g^{-1} wet)	4	4	5	2	2	1	3	4	5	5	6	5	46
tail muscle glycogen (mg.g^{-1} wet)	4	5	5	6	4	4	3	6	5	6	5	5	58
Hepatopancreas lipid (% wet)	3	5	5	3	4	4	2	5	6	3	6	5	51
tail muscle protein (% dry)	4	5	5	6	5	-	3	6	6	6	6	-	52
CL: weight ratio	4	4	5	6	5	4	3	6	6	6	6	5	60
HSI (% wet)	4	5	5	6	5	4	3	6	6	6	6	5	61
Hepatopancreas water (%)	4	5	5	6	5	4	3	6	6	6	6	5	61
tail muscle water (%)	4	5	5	6	5	4	3	6	6	6	6	5	61
tail muscle δ^{15}N (‰)	4	5	5	6	5	4	3	6	6	6	6	5	61
tail muscle δ^{13}C (‰)	4	5	5	6	5	4	3	6	6	6	6	5	61
tail muscle C: N ratio (wt)	4	5	5	6	5	4	3	6	6	6	6	5	61
Hepatopancreas δ^{15}N (‰)	3	3	3	2	2	1	3	3	3	3	3	5	34
Hepatopancreas δ^{13}C (‰)	3	3	3	2	2	1	3	3	3	3	3	5	34
Hepatopancreas C: N(wt)	3	3	3	2	2	1	3	3	3	3	3	5	34

Table A.2 Number of observations of female *N. norvegicus* in each unfed and unfed group at each week point for each parameter measured, as used in Chapter 3.

	Fed				unfed					
Measurement	**-2**	**0**	**12**	**20**	**4**	**8**	**12**	**16**	**20**	**n**
Carapace length (mm)	6	6	2	3	6	6	6	6	6	47
GSI (%)	6	6	2	3	6	6	6	6	6	47
Hepatopancreas copper (µg.g^{-1} wet)	6	6	2	3	6	6	6	6	6	47
Hepatopancreas glycogen (mg.g^{-1} wet)	3	4	2	2	3	4	6	3	3	30
tail muscle glycogen (mg.g^{-1} wet)	4	6	2	3	6	6	6	6	6	45
Hepatopancreas lipid (% wet)	3	5	2	3	5	5	5	4	5	37
CL: weight ratio	6	6	2	3	6	6	6	6	6	47
HSI (% wet)	6	6	2	3	6	6	6	6	6	47
Hepatopancreas water (%)	6	6	2	3	6	6	6	6	6	47
tail muscle water (%)	6	6	2	3	6	6	6	6	6	47
tail muscle δ^{15}N (‰)	6	6	2	3	6	6	6	6	6	47
tail muscle δ^{13}C (‰)	6	6	2	3	6	6	6	6	6	47
tail muscle C: N ratio (wt)	6	6	2	3	6	6	6	6	6	47

Table A.3 Number of observations for each measured parameter of Female (F) and Male (M) *N. norvegicus* taken from the Clyde Sea Area (CSA) and North Minch (NM) caught over various months, as used in Chapter 5.

		HSI		Water		Copper		Lipids		C:N ratio	
	sex	F	M	F	M	F	M	F	M	F	M
CSA	Feb-09	0	0	15	9	8	5	11	6	10	10
	Mar-09	13	13	13	13	12	12	5	6		
	Apr-09	9	14	1	3	1	3	0	0	7	10
	May-09	11	14	11	14	10	13	8	6		
	Jun-09	13	13	13	13	13	12	9	5	10	9
	Jul-09	15	15	15	15	14	15	6	6		
	Aug-09	14	13	14	13	14	13	7	7	10	10
	Sep-09	9	8	11	8	10	6	6	6		
	Oct-09	0	0	13	15	10	15	5	6	10	10
	Nov-09	14	13	14	13	10	13	5	4		
	Dec-09	0	0	12	13	9	12	4	7	8	10
	total	**98**	**103**	**132**	**129**	**111**	**119**	**66**	**59**	**55**	**59**
NM	Feb-09			8	10	5	10	6	5	10	10
	Apr-09			5	10	4	10	2	6	5	10
	Jun-09			10	10	10	10	6	6	10	10
	Aug-09			10	10	10	10	6	6	10	10
	Oct-09			8	9	7	9	3	6	8	9
	Dec-09			10	10	10	10	6	6	10	10
	total			**51**	**59**	**46**	**59**	**29**	**35**	**53**	**59**

Table A.4 Number of Male and Female *N. norvegicus* from the CSA and NM caught over various months used for Stable Isotope analysis as used in Chapter 6.

	CSA		NM	
	Male	Female	Male	Female
Feb-09	10	10	11	10
Apr-09	10	9	10	10
Jun-09	10	10	10	10
Aug-09	9	11	10	10
Oct-09	10	10	9	10
Dec-09	10	10	10	10

Table A.5 Number of other species caught in June 2010 used for Stable Isotope Analysis in Chapter 6.

	CSA	NM
lesser spotted dog fish	1	3
Long wrough Dab	6	6
Norway lobster	10	10
Queen scallop	6	3
Squat Lobster	5	6
Whiting	6	6

Table A.6 Number of Male and Female *N. norvegicus* from the CSA and NM caught over various months used for Fatty Acid Analysis as used in Chapter 7.

	CSA		NM	
	Male	Female	Male	Female
Feb-09	3	3	2	3
Apr-09			3	3
Jun-09	3	3	3	3
Aug-09	3	3	3	3
Oct-09	3	2	2	3
Dec-09	3	2	3	3

References

Ackman, R. G. & Eaton, C. A. (1967) Fatty acid composition of the Decapod shrimp *Pandalus borealis* in relation to that of Euphausid, M*eganyctiphanes norvegica*. *Journal of the Fisheries Research Board of Canada* 24**,** 467-471.

Ackman, R. G. & Hooper, S. N. (1973) Non-Methylene-Interrupted Fatty acids in lipids of shallow water marine invertebrates: a comparison of two molluscs (*Littorina littorea and Lunatia triseriata*) with the sand shrimp (*Crangon septemspinosus*). *Comparative Biochemistry and Physiology* 46B**,** 153-165.

Adams, S. M., Kimmel, B. L. & Ploskey, G. R. (1983) Sources of organic matter for reservoir fish production: A trophic-dynamics analysis. *Canadian Journal of Fisheries and Aquatic Sciences* 40**,** 1480-1495.

Adey, J. M. (2007) Aspects of the sustainability of creel fishing for Norway lobster, *Nephrops norvegicus* (L.), on the west coast of Scotland. PhD thesis. *University of Glasgow.*

Afonso-Dias, M. & Bailey, N. (1998) Estimates of Nephrops female length at first maturity from some Scottish stocks. *In: Report of the study group on Life Histories of Nephrops,* Appendix 9. ICES CM 1998/G:09 (mimeo).

Aguzzi, J., Company, J. B. & Sarda, F. (2007) The activity rhythm of berried and unberried females of *Nephrops norvegicus* (Decapoda, Nephropidae). *Crustaceana* 80**,** 1121-1134.

Aiken, D. E. & Waddy, S. L. (1980) Reproductive biology. in COBB, J. S. & PHILLIPS, B. F. (Eds.) *The biology and management of lobsters. Volume 1: Physiology and behaviour. Chapt. 2*. Academic Press. 215-276

Albalat, A., Sinclair, S., Laurie, J., Taylor, A. & Neil, D. (2010) Targeting the live market: Recovery of Norway lobsters *Nephrops norvegicus* (L.) from trawl-capture as assessed by stress-related parameters and nucleotide breakdown. *Journal of Experimental Marine Biology and Ecology* 395**,** 206-214.

Alcaraz, M. & Sarda, F. (1981) Oxygen-consumption by *Nephrops norvegicus* (L), (Crustacea, Decapoda) in relationship with its moulting stage. *Journal of Experimental Marine Biology and Ecology* 54**,** 113-118.

Alekseev, V. R. & Starobogatov, Y. I. (1996) Types of diapause in Crustacea: definitions, distribution, evolution. *Hydrobiologia* 320**,** 15-26.

Álvarez Fernández, I., Sardá , F., Rotllant, G., Verísimo, P. & Fernández, L. (2005) Role of lipid, protein, carbohydrate and nucleic acid as indicators of the moulting cycle of Norway lobster (*Nephrops norvegicus*). *ASLO Summer Meeting 2005, Santiago de Compostela, Spain, 19-24 June 2005*.

Angilletta, M. J., Niewiarowski, P. H. & Navas, C. A. (2002) The evolution of thermal physiology in ectotherms. *Journal of Thermal Biology* 27**,** 249-268.

Auerswald, L., Pape, C., Stuebing, D., Lopata, A. & Meyer, B. (2009) Effect of short-term starvation of adult Antarctic krill, *Euphausia superba*, at the onset of summer. *Journal of Experimental Marine Biology and Ecology* 381**,** 47-56.

Avarre, J. C., Michelis, R., Tietz, A. & Lubzens, E. (2003) Relationship between vitellogenin and vitellin in a marine shrimp (*Penaeus semisulcatus*) and molecular characterization of vitellogenin complementary DNAs. *Biology of Reproduction* 69**,** 355-364.

Baden, S. P., Depledge, M. H. & Hagerman, L. (1994) Glycogen depletion and altered copper and manganese handling in *Nephrops norvegicus* following starvation and exposure to hypoxia. *Marine Ecology Progress Series* 103**,** 65-72.

Baden, S. P., Pihl, L. & Rosenberg, R. (1990) Effects of oxygen depletion on the ecology, blood physiology and fishery of the Norway lobster *Nephrops norvegicus*. *Marine Ecology Progress Series* 67**,**141-155.

Bailey, N. (1984) Some aspects of reproduction in *Nephrops*. *International Council for the Exploration of the Sea C.M.* 1984/K:33**,** 1-39.

Bannon, R. O. & Roman, C. T. (2008) Using stable isotopes to monitor anthropogenic nitrogen inputs to estuaries. *Ecological Applications* 18**,** 22-30.

Barclay, M. C., Dall, W. & Smith, D. M. (1983) Changes in lipid and protein during starvation and the moulting cycle in the tiger prawn, *Penaeus esculentus* Haswell. *Journal of Experimental Marine Biology and Ecology* 68**,** 229-244.

Barnes, C., Jennings, S. & Barry, J. T. (2009) Environmental correlates of large-scale spatial variation in the $\delta^{13}C$ of marine animals. *Estuarine Coastal and Shelf Science* 81**,** 368-374.

Bauer, R. T. (1986) Phylogenetic trends in sperm transfer and storage complexity in Decapod Crustaceans. *Journal of Crustacean Biology* 6**,** 313-325.

Baxter, J. M., Boyd, I. L., Cox, M., Cunningham, L., Holmes, P. & Moffat C.F. (2008) Scotland's Seas: Toward Understanding their State. Fisheries Research Services, Aberdeen.

Bednekoff, P. A. & Houston, A. I. (1994) Optimizing fat reserves over the entire winter: a dynamic model. *Oikos* 71**,** 408-415.

Beevers, N. D., Kilbride, E., Atkinson, R. J. A. & Neil, D. M. (2012) A re-evaluation of *Hematodinium* infection seasonality in the Firth of Clyde Norway lobster (*Nephrops norvegicus*) population, Scotland. *Diseases of Aquatic Organisms In press*.

Bell, M. C., Redant, F. & Tuck, I. D. (2006) *Nephrops* Species. in PHILLIPS, B. F. (Ed.) *Lobster. biology, management, aquaculture and fisheries.* Blackwell Scientific Publishing. 412-461

Blanck, H. M., Bowman, B. A., Cooper, G. R., Myers, G. L. & Miller, D. T. (2003) Laboratory issues: use of nutritional biomarkers. *Journal of Nutrition* 133**,** 888S-894S.

Boag, B., Neilson, R. & Scrimgeour, C. M. (2006) The effect of starvation on the planarian *Arthurdendyus triangulatus* (Tricladida: Terricola) as measured by stable isotopes. *Biology and Fertility of Soils* 43**,** 267-270.

Boutton, T. W. (1991) Stable Carbon Isotope Ratios of Natural Materials: II. Atmospheric, Terrestrial, Marine, and Freshwater Environments. in COLEMAN, D. C. (Ed.) *Carbon Isotope Techniques. Chapt. 11*. Academic Press. 173-185

Briggs, R. P. (1995) Variability in northwest Irish Sea *Nephrops* populations. *Fisheries Research* 23**,** 175-187.

Brito, H. F. V., Lange, R. R., Pachaly, J. R. & Deconto, I. (2010) Determination of the basal metabolic rate in agoutis, *Dasyprocta azarae*, by indirect calorimetry. *Pesquisa Veterinaria Brasileira* 30**,** 471-478.

Budge, S. M., Iverson, S. J., Bowen, W. D. & Ackman, R. G. (2002) Among- and within-species variability in fatty acid signatures of marine fish and invertebrates on the Scotian Shelf, Georges Bank, and southern Gulf of St. Lawrence. *Canadian Journal of Fisheries and Aquatic Sciences* 59**,** 886-898.

Budge, S. M., Iverson, S. J. & Koopman, H. N. (2006) Studying trophic ecology in marine ecosystems using fatty acids: a primer on analysis and interpretation. *Marine Mammal Science* 22**,** 759-801.

Cabana, G. & Rasmussen, J. B. (1996) Comparison of aquatic food chains using nitrogen isotopes. *Proceedings of the National Academy of Sciences of the United States of America* 93**,** 10844-10847.

Campbell, N., Allan, L., Weetman, A. & Dobby, H. (2009) Investigating the link between *Nephrops norvegicus* burrow density and sediment composition in Scottish waters. *ICES Journal of Marine Science* 66**,** 2052-2059.

Carlier, A., Riera, P., Amouroux, J. M., Bodiou, J. Y., Desmalades, M. & Gremare, A. (2008) Food web structure of two Mediterranean lagoons under varying degree of eutrophication. *Journal of Sea Research* 60**,** 264-275.

Chang, E. S. (1995) Physiological and biochemical changes during the molt cycle in decapod crustaceans: an overview. *Journal of Experimental Marine Biology and Ecology* 193**,** 1-14.

Chapelle, G., Peck, L. S. & Clarke, A. (1994) Effects of feeding and starvation on the metabolic rate of the necrophagous Antarctic amphipod *Waldeckia obesa* (Chevreux, 1905). *Journal of Experimental Marine Biology and Ecology* 183**,** 63-76.

Chapman, C. J. & Bailey, N. (1987) Recent progress in Norway lobster research. *Developments in fisheries research in Scotland.***,** 99-111.

Chapman, C. J. & Howard, F. G. (1979) Field observations on the emergence rhythm of the Norway Lobster *Nephrops norvegicus*, using different methods. *Marine Biology* 51**,** 157-165.

Chapman, C. J. & Rice, A. L. (1971) Some direct observations on ecology and behaviour of Norway Lobster *Nephrops norvegicus*. *Marine Biology* 10**,** 321-329.

Cherel, Y., Hobson, K. A., Bailleul, F. R. & Groscolas, R. (2005) Nutrition, physiology, and stable isotopes: New information from fasting and molting penguins. *Ecology* 86**,** 2881-2888.

Chrisite, W. W. (1982) *Lipid analysis*, Pergamon Press, Oxford.

Clarke, A. (1979) Lipid content and composition of the pink shrimp, *Pandalus montagui* (Leach) (Crustacea: Decapoda). *Journal of Experimental Marine Biology and Ecology* 38**,** 1-17.

Comoglio, L., Goldsmit, J. & Amin, O. (2008) Starvation effects on physiological parameters and biochemical composition of the hepatopancreas of the southern king crab *Lithodes santolla* (Molina, 1782). *Revista De Biologia Marina Y Oceanografia* 43**,** 345-353.

Comoglio, L., Smolko, L. & Amin, O. (2005) Effects of starvation on oxygen consumption, ammonia excretion and biochemical composition of the hepatopancreas on adult males of the False Southern King crab *Paralomis granulosa* (Crustacea, Decapoda). *Comparative Biochemistry and Physiology B: Biochemistry & Molecular Biology* 140**,** 411-416.

Cook, E. J., Shucksmith, R., Orr, H., Ashton, G. V. & Berge, J. (2010) Fatty acid composition as a dietary indicator of the invasive caprellid, *Caprella mutica* (Crustacea: Amphipoda). *Marine Biology* 157**,** 19-27.

Cooper, M. H., Iverson, S. J. & Rouvinen-Watt, K. (2006) Metabolism of dietary cetoleic acid (22:1n-11) in mink (*Mustela vison*) and gray seals (*Halichoerus grypus*) studied using radiolabeled fatty acids. *Physiological and Biochemical Zoology* 79**,** 820-829.

Cuzon, G., Cahu, C., Aldrin, J. F., Messager, J. L., Stephan, G. & Mevel, M. (1980) Starvation effect on metabolism of *Penaeus japonicus*. *Proceedings of the Annual Meeting of the World Mariculture Society* 11**,** 410-423.

Dall, W. (1974) Indexes of nutritional state in the western rock lobster, *Panulirus longipes* (Milne-Edwards). I. Blood and tissue constituents and water-content. *Journal of Experimental Marine Biology and Ecology* 16**,** 167-180.

Dall, W. (1975) Indexes of nutritional state in western rock lobster, *Panulirus longipes* (Milne Edwards). II. Gastric fluid constituents. *Journal of Experimental Marine Biology and Ecology* 18**,** 1-18.

Dall, W. (1981) Lipid absorption and utilization in the Norwegian Lobster, *Nephrops norvegicus* (L.). *Journal of Experimental Marine Biology and Ecology* 50**,** 33-45.

Dall, W. & Moriarty, D. J. W. (1983) Functional aspects of nutrition and digestion. in MANTEL, L. H. (Ed.) The Biology of Crustacea Volume 5. Internal Anatomy and Physiological Regulation. Chapt. 4. Academic Press. 215-261

Dendinge, J. E. & Schatzle, F. C. (1973) Carbohydrate metabolism in striped shore crab, *Pachygrapsus crassipes*. II. glycolytic rates of muscle, gill and hepatopancreas. *Comparative Biochemistry and Physiology* 46**,** 699-708.

Depledge, M. H. & Bjerregaard, P. (1989) Hemolymph protein composition and copper levels in decapod crustaceans. *Helgolander Meeresunters* 43**,** 207-223.

Dickson, G. W. & Giesy, J. P. (1982) The effects of starvation on muscle phosphoadenylate concentrations and adenylate energy charge of surface and cave crayfish. *Comparative Biochemistry and Physiology A - Physiology* 71**,** 357-361.

Engel, D. W. & Brouwer, M. (1993) Crustaceans as models for metal metabolism: I. Effects of the molt cycle on blue crab metal metabolism and metallothionein. *Marine Environmental Research* 35**,** 1-5.

Evershed, R. P., Bull, I. D., Corr, L. T., Crossman, Z. M., Van Dongen, B. E., Evans, C. J., Jim, S., Mottram, H. R., Mukherjee, A. J. & Pancost, R. D. (2007) Compund-specific stable isotope analysis in ecology and paleoecology. in MICHENER. R & LAJTHA. K (Eds.) *Stable Isotopes in Ecology and Environmental Science. Chapt. 14*. Blackwell Publishing. 480-540

Factor, J. R. (1995) The Digestive System. in FACTOR, J. R. (Ed.) *Biology of Lobsters Homarus americanus. Chapt. 15*. Academic Press, Inc. 395-440

Falk-Petersen, S., Hagen, W., Kattner, G., Clarke, A. & Sargent, J. (2000) Lipids, trophic relationships, and biodiversity in Arctic and Antarctic krill. *Canadian Journal of Fisheries and Aquatic Sciences* 57**,** 178-191.

Fanelli, E., Papiol, V., Cartes, J. E., Rumolo, P., Brunet, C. & Sprovieri, M. (2011) Food web structure of the epibenthic and infaunal invertebrates on the Catalan slope (NW Mediterranean): Evidence from $\delta^{13}C$ and $\delta^{15}N$ analysis. *Deep-Sea Research Part I Oceanographic Research Papers* 58**,** 98-109.

FAO, Fisheries and Aquaculture Information and Statistics Service (2009) Total fishery production 1950-2007. FISHSTAT Plus - Universal software for fishery statistical time series online. *Food and Agriculture Organization of the United Nations* Available at: http://www.fao.org/fi/statist/FISOFT/FISHPLUS.asp

Farmer, A. S. D. (1974) Development of external sexual characters of *Nephrops norvegicus* (L.) (Decapoda: Nephropidae). *Journal of Natural History* 8**,** 241-255.

Fernandez, I., Oliva, M., Carrillo, O. & Wormhoudt, A. V. (1997) Digestive enzyme activities of *Penaeus notialis* during reproduction and moulting cycle. *Comparative Biochemistry and Physiology a-Molecular and Integrative Physiology* 118**,** 1267-1271.

Field, R. H. & Appleton, P. L. (1995) A *Hematodinium*-like dinoflagellate infection of the Norway lobster *Nephrops norvegicus*: observations on pathology and progression of infection. *Diseases of Aquatic Organisms* 22**,** 115-128.

Field, R. H., Chapman, C. J., Taylor, A. C., Neil, D. M. & Vickerman, K. (1992) Infection of the Norway lobster *Nephrops norvegicus* by a *Hematodinium*-like species of dinoflagellate on the west coast of Scotland. *Diseases of Aquatic Organisms* 13**,** 1-15.

Field, R. H., Hills, J. M., Atkinson, R. J. A., Magill, S. & Shanks, A. M. (1998) Distribution and seasonal prevalence of *Hematodinium* sp. infection of the Norway lobster (*Nephrops norvegicus*) around the west coast of Scotland. *ICES Journal of Marine Science* 55**,** 846-858.

Folch, J., Lees, M. & Sloane Stanley, G. H. (1957) A simple method for the isolation and purification of total lipids from animal tissues. *Journal of Biological Chemistry* 226**,** 497-509.

Fry, B. (1996) $^{13}C/^{12}C$ fractionation by marine diatoms. *Marine Ecology Progress Series* 134**,** 283-294.

Galloway, T. S. (2006) Biomarkers in environmental and human health risk assessment. *Marine Pollution Bulletin* 53**,** 606-613.

Garland, J. & Uglow, R. (2011) Effect of temperature and body size on long-term emersion and re-immersion responses of the American lobster, *Homarus americanus*. *9th International conference and workshop on Lobster biology and management,* 58 http://www.imr.no/filarkiv/2011/10/9thicwlbok_web_ny.pdf/en

Garthe, S., Camphuysen, C. J. & Furness, R. W. (1996) Amounts of discards by commercial fisheries and their significance as food for seabirds in the North Sea. *Marine Ecology Progress Series* 136**,** 1-11.

Gascuel, D., Guenette, S. & Pauly, D. (2011) The trophic-level-based ecosystem modelling approach: theoretical overview and practical uses. *ICES Journal of Marine Science* 68**,** 1403-1416.

Gaye-Siessegger, J., Focken, U., Abel, H. & Becker, K. (2007) Starvation and low feeding levels result in an enrichment of ^{13}C in lipids and ^{15}N in protein of Nile tilapia *Oreochromis niloticus* L. *Journal of Fish Biology* 71**,** 90-100.

Gibson, R. & Barker, P. L. (1979) The decapod hepatopancreas. *Oceanography and Marine Biology an Annual Review* 17**,** 285-346.

Gomez-Campos, E., Borrell, A. & Aguilar, A. (2011) Nitrogen and carbon stable isotopes do not reflect nutritional condition in the striped dolphin. *Rapid Communications in Mass Spectrometry* 25**,**1343-1347.

Govind, C. K. (1995) Muscles and their innervations. in FACTOR, J. R. (Ed.) *Biology of Lobsters Homarus americanus. Chapt. 12*. Academic Press, Inc. 291-312

Gramitto, M. E. (1998) Molt pattern identification through gastrolith examination on *Nephrops norvegicus* (L.) in the Mediterranean Sea. *Scientia Marina* 62**,** 17-23.

Guppy, M. & Withers, P. (1999) Metabolic depression in animals: physiological perspectives and biochemical generalizations. *Biological Reviews* 74**,** 1-40.

Hagerman, L. (1983) Hemocyanin concentration of juvenile lobsters (*Homarus gammarus*) in relation to molting cycle and feeding conditions. *Marine Biology* 77**,** 11-17.

Hairston, N. G. & Caceres, C. E. (1996) Distribution of crustacean diapause: micro- and macroevolutionary pattern and process. *Hydrobiologia* 320**,** 27-44.

Hamilton, K. M., Shaw, P. W. & Morritt, D. (2010) Physiological responses of three crustacean species to infection by the dinoflagellate-like protest *Hematodinium* (Alveolata: Syndinea). *Journal of Invertebrate Pathology* 105**,** 194-196.

Hardy, D., Dutil, J. D., Godbout, G. & Munro, J. (2000) Survival and condition of hard shell male adult snow crabs (*Chionoecetes opilio*) during fasting at different temperatures. *Aquaculture* 189**,** 259-275.

Haubert, D., Langel, R., Scheu, S. & Ruess, L. (2005) Effects of food quality, starvation and life stage on stable isotope fractionation in Collembola. *Pedobiologia* 49**,** 229-237.

Hawkins, A. J. S. (1995) Effects of temperature change of ectoterm metabolism and evolution: Metabolic and physiological interactions underlying the superiority of multi-locus heterozygotes in heterogeneous environments. *Journal of Thermal Biology* 20**,** 23-33.

Heath, J. R. & Barnes, H. (1970) Some changes in biochemical composition with season and during the molting cycle of the common shore crab *Carcinus maenas* (L.). *Journal of Experimental Marine Biology and Ecology* 5**,** 199-233.

Heaton, T. H. E. (1986) Isotopic studies of nitrogen pollution in the hydrosphere and atmosphere: a review. *Chemical Geology* 59**,** 87-102.

Hervant, F., Mathieu, J. & Barre, H. (1999) Comparative study on the metabolic responses of subterranean and surface-dwelling amphipods to long-term starvation and subsequent refeeding. *Journal of Experimental Biology* 202**,** 3587-3595.

Hill, J. M. (2008) Structure and flow of carbon and nitrogen in the Western Irish Sea *Nephrops norvegicus* Fishery: A stable isotope approach. PhD thesis. *University of London.*

Hu, M., Wang, Y., Tsang, S. T., Cheung, S. G. & Shin, P. K. S. (2011) Effect of starvation on the energy budget of two Asian horseshoe crab species: *Tachypleus tridentatus* and *Carcinoscorpius rotundicauda* (Chelicerata: Xiphosura). *Marine Biology* 158**,** 1591-1600.

Hyslop, E. J. (1980) Stomach contents analysis- a review of methods and their application. *Journal of Fish Biology* 17**,** 411-429.

I.M.B.C., U.M.B.S.M. & I.R.P.E.M. (1994) *Nephrops norvegicus*: stock variability and assessment in relation to fishing pressure and environmental factors. Final report to EC, Contract XIV-1/MED/91/003. *Programme co-ordinator Eleftheriou, A. (Institute of Marine Biology of Crete, IMBC), senior editor Smith, C.J. (IMBC), Scottish coordinator Atkinson, R.J.A. (University Marine Biological Station Millport, UMBSM), Italian co-ordinator Froglia, C. (Istituto di Ricerche sulla Pesca Marittima, Ancona, IRPEM).*

ICES (2011) ICES Nephrops stock advice 2011 Division VIa 5.4.33, 204-234. http://www.ices.dk/committe/acom/comwork/report/2011/2011/Neph-Via.pdf.

Iverson, S. J., Stewart, B. S. & Yochem, P. K. (2003) Captive validation and calibration of fatty acid signatures in blubber as indicators of prey in Hawaiian monk seal diet. *NOAA Technical Report, San Diego, CA* 22.

Jennings, S., Barnes, C., Sweeting, C. J. & Polunin, N. V. C. (2008) Application of nitrogen stable isotope analysis in size-based marine food web and macroecological research. *Rapid Communications in Mass Spectrometry* 22**,** 1673-1680.

Jennings, S., Greenstreet, S. P. R., Hill, L., Piet, G. J., Pinnegar, J. K. & Warr, K. J. (2002) Long-term trends in the trophic structure of the North Sea fish community: evidence from stable-isotope analysis, size-spectra and community metrics. *Marine Biology* 141**,** 1085-1097.

Jennings, S. & Warr, K. J. (2003) Smaller predator-prey body size ratios in longer food chains. *Proceedings of the Royal Society of London Series B: Biological Sciences* 270**,** 1413-1417.

Jones, P. L. & Obst, J. H. (2000) Effects of starvation and subsequent refeeding on the size and nutrient content of the hepatopancreas of *Cherax destructor* (Decapoda: Parastacidae). *Journal of Crustacean Biology* 20**,** 431-441.

Joseph, J. D. (1982) Lipid composition of marine and estuarine invertebrates. Part II: Mollusca. *Progress in Lipid Research* 21**,** 109-153.

Kaiser, M. J. & Hiddink, J. G. (2007) Food subsidies from fisheries to continental shelf benthic scavengers. *Marine Ecology Progress Series* 350**,** 267-276.

Keltz, S. & Bailey, N. (2010) Fish and Shellfish Stocks 2010. *Marine Scotland Science publication. ISSN 2044-0340*.

Kendall, C., Elliott, E. M. & Wankel, S. D. (2007) Tracing anthropogenic inputs of nitrogen to ecosystems. in MICHENER, R. H. & LAJHA, K. (Eds.) *Stable Isotopes in Ecology and Environmental Science. Chapt. 12*. Blackwell publishing. 375-449

Kharlamenko, V. I., Zhukova, N. V., Khotimchenko, S. V., Svetashev, V. I. & Kamenev, G. M. (1995) Fatty acids as markers of food sources in a shallow-water hydrothermal ecosystem (Kraternaya Bight, Yankich Island, Kurile Islands). *Marine Ecology Progress Series* 120**,** 231-241.

Kohn, E. C., Azad, N., Annunziata, C., Dhamoon, A. S. & Whiteley, G. (2007) Proteomics as a tool for biomarker discovery. *Disease Markers* 23**,** 411-417.

Koulman, A., Lane, G. A., Harrison, S. J. & Volmer, D. A. (2009) From differentiating metabolites to biomarkers. *Analytical and Bioanalytical Chemistry* 394**,** 663-670.

Krang, A. S. & Rosenqvist, G. (2006) Effects of manganese on chemically induced food search behaviour of the Norway lobster, *Nephrops norvegicus* (L.). *Aquatic Toxicology* 78**,** 284-291.

Krasne, F. B. & Wine, J. J. (1975) Extrinsic modulation of crayfish escape behaviour. *Journal of Experimental Biology* 63**,** 433-450.

Lagerspetz, K. Y. H. & Vainio, L. A. (2006) Thermal behaviour of crustaceans. *Biological Reviews* 81**,** 237-258.

Le Loc'h, F. & Hily, C. (2005) Stable carbon and nitrogen isotope analysis of *Nephrops norvegicus* / *Merluccius merluccius* fishing grounds in the Bay of Biscay (Northeast Atlantic). *Canadian Journal of Fisheries and Aquatic Sciences* 62**,** 123-132.

Lindeman, R. L. (1942) The trophic-dynamic aspect of ecology. *Ecology* 23**,** 399-418.

Loo, L. O., Baden, S. P. & Ulmestrand, M. (1993) Suspension feeding in adult *Nephrops norvegicus* (L.) and *Homarus gammarus* (L.) (Decapoda). *Netherlands Journal of Sea Research* 31**,** 291-297.

Macleod, R., Clark, J. & Cresswell, W. (2008) The starvation-predation risk trade-off, body mass and population status in the Common Starling *Sturnus vulgaris*. *Ibis* 150**,** 199-208.

Main, J. & Sangster, G. I. (1985) The behaviour of the Norway lobster, *Nephrops norvegicus* (L.), during trawling. *Scottish Fisheries Research Report***,** 1-23.

Marshall, J. D., Brooks, J. R. & Lajha, K. (2007) Sources of variation in the stable isotopic composition of plants. in MICHENER, R. H. & LAJHA, K. (Eds.) *Stable Isotopes in Ecology and Environmental Science. Chapt. 2*. Blackwell publishing. 22-60

Martin, G. G. & Hose, J. E. (1995) Circulation, the Blood, and Disease. in FACTOR, J. R. (Ed.) *Biology of Lobsters Homarus americanus. Chapt. 17*. Academic Press, Inc. 465-495

Maynou, F. & Sarda, F. (1997) *Nephrops norvegicus* population and morphometrical characteristics in relation to substrate heterogeneity. *Fisheries Research* 30**,** 139-149.

Maynou, F. & Sarda, F. (2001) Influence of environmental factors on commercial trawl catches of *Nephrops norvegicus* (L.). *ICES Journal of Marine Science* 58**,** 1318-1325.

Mccue, M. D. (2010) Starvation physiology: Reviewing the different strategies animals use to survive a common challenge. *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology* 156**,** 1-18.

Mccue, M. D. & Pollock, E. D. (2008) Stable isotopes may provide evidence for starvation in reptiles. *Rapid Communications in Mass Spectrometry* 22**,** 2307-2314.

Mcleod, L. E., Carter, C. G. & Johnston, D. J. (2004) Changes in the body composition of adult male southern rock lobster, *Jasus edwardsii*, during starvation. *Journal of Shellfish Research* 23**,** 257-264.

Mcquaid, N. & Briggs, R. P. (2004) Sexual maturity of male *Nephrops norvegicus* (L.) in the Irish Sea. *Working Document to WGNEPH* ICES CM 2004/ACFM: 19 (mimeo): Appendix 6**,** 363-73.

Mente, E. (2010) Survival, food consumption and growth of Norway lobster (*Nephrops norvegicus*) kept in laboratory conditions. *Integrative Zoology* 5**,** 256-263.

Mente, E., Carter, C. G., Barnes, R. S. & Karapanagiotidis, I. T. (2011) Protein synthesis in wild-caught Norway lobster (*Nephrops norvegicus* L.). *Journal of Experimental Marine Biology and Ecology* 409**,** 208-214.

Mente, E., Karapanagiotidis, I. T., Logothetis, P., Vafidis, D., Malandrakis, E., Neofitou, N., Exadactylos, A. & Stratakos, A. (2009) The reproductive cycle of Norway lobster. *Journal of Zoology* 278**,**324-332.

Michener, R. H. & Kaufman, L. (2007) Stable isotope ratios as tracers in marine food webs: An update. in MICHENER, R. H. & LAJHA, K. (Eds.) *Stable Isotopes in Ecology and Environmental Science. Chapt. 9*. Blackwell publishing. 238-282

Milligan, R. J., Albalat, A., Atkinson, R. J. A. & Neil, D. M. (2009) The effects of trawling on the physical condition of the Norway lobster *Nephrops norvegicus* in relation to seasonal cycles in the Clyde Sea Area. *ICES Journal of Marine Science* 66**,** 488-494.

Milligan, R. J. & Neil, D. (in prep) Bycatch composition in an MSC-accredited *Nephrops* trawl fishery and implications for future management.

Minagawa, M. & Wada, E. (1984) Stepwise enrichment of ^{15}N along foodchains-further evidence and the relation between $\delta^{15}N$ and animal age. *Geochimica et Cosmochimica Acta* 48**,** 1135-1140.

Mohamad, F. (2008) Uptake of manganese into the exoskeleton of the swimming crab *Liocarcinus depurator* (L.) in relation to biomonitoring and bioabsorption. PhD thesis. *University of Glasgow.*

Montoya, J. P. (2007) Natural abundance of ^{15}N in marine planktonic ecosystems. in MICHENER, R. H. & LAJHA, K. (Eds.) *Stable Isotopes in Ecology and Environmental Science. Chapt. 7*. Blackwell publishing. 176-201

Moss, S. M. (1994) Use of nucleic acids as indicators of growth in juvenile white shrimp, *Penaeus vannamei*. *Marine Biology* 120**,** 359-367.

Neil, D. M. & Ansell, A. D. (1995) The orientation of tail-flip escape swimming in decapod and mysid crustaceans. *Journal of the Marine Biological Association of the United Kingdom* 75**,** 55-70.

Neiland, K. A. & Scheer, B. T. (1953) The influence of fasting and of sinus gland removal on body composition of *Hemigrapsus nudus*. (Part V of the hormonal regulation of metabolism in Crustaceans. *Physiol Comp Et Oecol* 3**,** 321-326.

Newell, R. C. & Bayne, B. L. (1973) A review on temperature and metabolic acclimation in intertidal marine invertebrates, from the 7th European Symposium on Marine Biology. *Netherlands Journal of Sea Research* 7**,** 421-433.

Newland, P. L. (1985) The control of escape behaviour in the Norway Lobster, *Nephrops norvegicus* (L). PhD Thesis. *University of Glasgow.*

Newland, P. L., Neil, D. M. & Chapman, C. J. (1992) Escape swimming in the Norway lobster. *Journal of Crustacean Biology* 12**,** 342-353.

Nier, A. O. & Gulbransen, E. A. (1939) Variations in the relative abundance of the carbon isotopes. *Journal of the American Chemical Society* 61**,** 697-698.

O.E.D. (2012) Oxford English Dictionary. www.oed.co.uk

Oakley, S. (1978) Food, feeding behaviour and some aspects of the ecology of *Nephrops norvegicus* in the Irish Sea. PhD thesis. *University of Liverpool.*

Odum, W. E. & Heald, E. J. (1975) The detritus-based food web of an estuarine community. in CRONIN, L. E. (Ed.) *Estuarine Research.* New York, Academic Press. 265-286

Pampoulie, C., Skirnisdottir, S., Hauksdottir, S., Olafsson, K., Eiriksson, H., Chosson, V., Hreggvidsson, G. O., Gunnarsson, G. H. & Hjorleifsdottir, S. (2011) A pilot genetic study reveals the absence of spatial genetic structure in Norway lobster (*Nephrops norvegicus*) on fishing grounds in Icelandic waters. *ICES Journal of Marine Science* 68**,** 20-25.

Parker, K. L., Barboza, P. S. & Stephenson, T. R. (2005) Protein conservation in female caribou (*Rangifer tarandus*): effects of decreasing diet quality during winter. *Journal of Mammalogy* 86**,** 610-622.

Parslow-Williams, P. J. (1998) Nutritional limitation in populations of the Norway lobster, *Nephrops norvegicus* (L.) in the Firth of Clyde, Scotland. PhD thesis. *University of Glasgow.*

Parslow-Williams, P. J., Atkinson, R. J. A. & Taylor, A. C. (2001) Nucleic acids as indicators of nutritional condition in the Norway lobster *Nephrops norvegicus*. *Marine Ecology Progress Series* 211**,**235-243.

Parslow-Williams, P. J., Goodheir, C., Atkinson, R. J. A. & Taylor, A. C. (2002) Feeding energetics of the Norway lobster, *Nephrops norvegicus* in the Firth of Clyde, Scotland. *Ophelia* 56**,** 101-120.

Pascual, C., Sanchez, A., Zenteno, E., Cuzon, G., Gabriela, G., Brito, R., Gelabert, R., Hidalgo, E. & Rosas, C. (2006) Biochemical, physiological, and immunological changes during starvation in juveniles of *Litopenaeus vannamei*. *Aquaculture* 251**,** 416-429.

Peterson, B. J. & Fry, B. (1987) Stable isotopes in ecosystem studies. *Annual Review of Ecology and Systematics* 18**,** 293-320.

Pond, D. W., Fallick, A. E., Stevens, C. J., Morrison, D. J. & Dixon, D. R. (2008) Vertebrate nutrition in a deep-sea hydrothermal vent ecosystem: Fatty acid and stable isotope evidence. *Deep-Sea Research Part I-Oceanographic Research Papers* 55**,** 1718-1726.

Pond, D. W., Gebruk, A., Southward, E. C., Southward, A. J., Fallick, A. E., Bell, M. V. & Sargent, J. R. (2000) Unusual fatty acid composition of storage lipids in the bresilioid shrimp *Rimicaris exoculata* couples the photic zone with MAR hydrothermal vent sites. *Marine Ecology Progress Series* 198**,** 171-179.

Post, D. M. (2002) Using stable isotopes to estimate trophic position: models, methods, and assumptions. *Ecology* 83**,** 703-718.

Powell, M. L. & Watts, S. A. (2010) Response to Long-term Nutrient Deprivation and Recovery in the Crayfishes *Procambarus clarkii* and *Procambarus zonangulus* (Crustacea, Decapoda): Component and Proximate Analyses. *Journal of the World Aquaculture Society* 41**,** 71-80.

Ramsay, K., Kaiser, M. J., Moore, P. G. & Hughes, R. N. (1997) Consumption of fisheries discards by benthic scavengers: utilization of energy subsidies in different marine habitats. *Journal of Animal Ecology* 66**,** 884-896.

Regnault, M. (1981) Respiration and ammonia excretion of the shrimp *Crangon crangon* L.: metabolic response to prolonged starvation. *Journal of Comparative Physiology* 141**,** 549-555.

Regnault, M. (1989) Glutamate-Dehydrogenase (GDH) in the cheliped muscle of the crab *Cancer pagurus* L. effect of a prolonged starvation. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology* 92**,** 721-725.

Rippeth, T. P. & Simpson, J. H. (1996) The frequency and duration of episodes of complete vertical mixing in the Clyde Sea. *Continental Shelf Research* 16**,** 933-947.

Ritar, A. J., Dunstan, G. A., Crear, B. J. & Brown, M. R. (2003) Biochemical composition during growth and starvation of early larval stages of cultured spiny lobster (*Jasus edwardsii*) phyllosoma. *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology* 136**,** 353-370.

Roots, C. (2006) *Hibernation,* London, Greenwood Press.

Rosa, R., Morais, S., Calado, R., Narciso, L. & Nunes, M. L. (2003) Biochemical changes during the embryonic development of Norway lobster, *Nephrops norvegicus*. *Aquaculture* 221**,** 507-522.

Rosa, R. & Nunes, M. L. (2002 a) Biochemical changes during the reproductive cycle of the deep-sea decapod *Nephrops norvegicus* on the south coast of Portugal. *Marine Biology* 141**,** 1001-1009.

Rosa, R. & Nunes, M. L. (2002 b) Changes in organ indices and lipid dynamics during the reproductive cycle of *Aristeus antennatus*, *Parapenaeus Longirostris*, and *Nephrops norvegicus* (Decapoda) from the portuguese south coast. *Crustaceana* 75**,** 1095-1105.

Rosa, R. & Nunes, M. L. (2003) Seasonal changes in nucleic acids, amino acids and protein content in juvenile Norway lobster (*Nephrops norvegicus*). *Marine Biology* 143**,** 565-572.

Rotllant, G., Ribes, E., Company, J. B. & Durfort, M. (2005) The ovarian maturation cycle of the Norway lobster *Nephrops norvegicus* (Linnaeus, 1758) (Crustacea, Decapoda) from the western Mediterranean Sea. *Invertebrate Reproduction and Development* 48**,** 161-169.

Sanchez-Paz, A., Garcia-Carreno, F., Muhlia-Almazan, A., Peregrino-Uriarte, A. B., Hernandez-Lopez, J. & Yepiz-Plascencia, G. (2006) Usage of energy reserves in crustaceans during starvation: Status and future directions. *Insect Biochemistry and Molecular Biology* 36**,** 241-249.

Sara, G., De Pirro, M., Sprovieri, M., Rumolo, P., Halldorsson, H. P. & Svavarsson, J. (2009) Carbon and nitrogen stable isotopic inventory of the most abundant demersal fish captured by benthic gears in southwestern Iceland (North Atlantic). *Helgoland Marine Research* 63**,** 309-315.

Sarda, F. & Valladares, F. J. (1990) Gastric evacuation of different foods by *Nephrops norvegicus* (Crustacea, Decapoda) and estimation of soft tissue ingested, maximum food intake and cannibalism in captivity. *Marine Biology* 104**,** 25-30.

Sargent, J. R. & Whittle, K. J. (1981) Lipids and Hydrocarbons in the Marine Food Web. in LONGHURST, A. R. (Ed.) *Analysis of Marine Ecosystems. Chapt. 17*. Academic Press. 491-534

Schatzle, F. C., Carpente, H. M., Rogers, M. R. & Sutko, J. L. (1973) Carbohydrate metabolism in striped shore crab, *Pachygrapsus crassipes* . I. Glycolytic enzymes of gill, hepatopancreas, heart and leg muscles. *Comparative Biochemistry and Physiology* 45**,** 393-405.

Schmidt-Nielsen, K. (1997) *Animal Physiology: adaptation and environment*, Cambridge University Press.

Semenina, E. E. & Tiunov, A. V. (2011) Trophic fractionation ($\Delta^{15}N$) in Collembola depends on nutritional status: A laboratory experiment and mini-review. *Pedobiologia* 54**,** 101-109.

Sharp, Z. (2006) Terminology, Standards, and Mass Spectrometry. in SHARP, Z. (Ed.) *Principles of Stable Isotope Geochemistry. Chapt. 2*. Prentice Hall. 15-39

Sigdel, T. K. & Sarwal, M. M. (2008) The proteogenomic path towards biomarker discovery. *Pediatric Transplantation* 12**,** 737-747.

Small, H. J., Wilson, S., Neil, D. M., Hagan, P. & Coombs, G. H. (2002) Detection of the parasitic dinoflagellate *Hematodinium* in the Norway lobster *Nephrops norvegicus* by ELISA. *Diseases of Aquatic Organisms* 52**,** 175-177.

Smith, R. S. M. (1987) The biology of larval and juvenile *Nephrops norvegicus* (L.) in the Firth of Clyde. PhD thesis. *University of Glasgow.*

Spilmont, N., Meziane, T., Seuront, L. & Welsh, D. T. (2009) Identification of the food sources of sympatric ghost shrimp (*Trypaea australiensis*) and soldier crab (*Mictyris longicarpus*) populations using a lipid biomarker, dual stable isotope approach. *Austral Ecology* 34**,** 878-888.

Spoek, G. L. (1974) The relationship between blood haemocyanin level, oxygen uptake, and the heart-beat and scaphognathite-beat frequencies in the lobster *Homarus gammarus*. *Netherlands Journal of Sea Research* 8**,** 1-26

Stentiford, G. D., Chang, E. S., Chang, S. A. & Neil, D. M. (2001 a) Carbohydrate dynamics and the crustacean hyperglycemic hormone (CHH): effects of parasitic infection in Norway lobsters (*Nephrops norvegicus*). *General and Comparative Endocrinology* 121**,** 13-22.

Stentiford, G. D. & Neil, D. M. (2011) Diseases of *Nephrops* and *Metanephrops*: a review. *Journal of Invertebrate Pathology* 106**,** 92-109.

Stentiford, G. D., Neil, D. M. & Atkinson, R. J. A. (2001 b) The relationship of *Hematodinium* infection prevalence in a Scottish *Nephrops norvegicus* population to season, moulting and sex. *ICES Journal of Marine Science* 58**,** 814-823.

Stentiford, G. D. & Shields, J. D. (2005) A review of the parasitic dinoflagellates *Hematodinium* species and *Hematodinium*-like infections in marine crustaceans. *Diseases of Aquatic Organisms* 66**,** 47-70.

Stephens, K., Sheldon, R. W. & Parsons, T. R. (1967) Seasonal variations in availability of food for benthos in a coastal environment. *Ecology* 48**,** 852-855.

Storch, V., Janssen, H. H. & Cases, E. (1982) The effects of starvation on the hepatopancreas of the coconut crab, *Birgus latro* (L.) (Crustacea: Decapoda). *Zoologischer Anzeiger* 208**,** 115-123.

Storey, K. B. & Storey, J. M. (1990) Metabolic rate depression and biochemical adaptation in anaerobiosis, hibernation and estivation. *Quarterly Review of Biology* 65**,** 145-174.

Stowasser, G., Pond, D. W. & Collins, M. A. (2009) Using fatty acid analysis to elucidate the feeding habits of Southern Ocean mesopelagic fish. *Marine Biology* 156**,** 2289-2302.

Styrishave, B. & Andersen, O. (2000) Seasonal variations in hepatopancreas fatty acid profiles of two colour forms of shore crabs, *Carcinus maenas*. *Marine Biology* 137**,** 415-422.

Sulzman, E. W. (2007) Stable isotope chemistry and measurement: a primer. in MICHENER, R. H. & LAJHA, K. (Eds.) *Stable Isotopes in Ecology and Environmental Science. Chapt. 1*. Blackwell publishing. 1-21

Symonds, D. J. (1972) The fishery for the Norway lobster *Nephrops norvegicus* off the northeast coast of England. Fishery Investigations Series II Marine Fisheries Great Britain Ministry of Agriculture Fisheries and Food 27**,** 1-35.

Talbot, P. & Helluy, S. (1995) Reproduction and embryonic development. in FACTOR, J. R. (Ed.) *Biology of Lobsters Homarus americanus. Chapt. 9*. Academic Press, Inc. 177-216

Taylor, A. C., Field, R. H. & Parslow-Williams, P. J. (1996) The effects of *Hematodinium* sp infection on aspects of the respiratory physiology of the Norway lobster, *Nephrops norvegicus* (L.). *Journal of Experimental Marine Biology and Ecology* 207**,** 217-228.

Taylor, H. H. & Anstiss, J. M. (1999) Copper and haemocyanin dynamics in aquatic invertebrates. *Marine and Freshwater Research* 50**,** 907-931.

Thebault, M. T. & Raffin, J. P. (1991) Seasonal variations in *Palaemon serratus* abdominal muscle metabolism and performance during exercise, as studied by ^{31}P NMR. *Marine Ecology Progress Series*74**,** 175-183.

Thomas, H. J. (1964) The spawning and fecundity of the Norway lobsters (*Nephrops norvegicus* L.) around the Scottish Coast. *Journal du Conseil / Conseil Permanent International pour l'Exploration de la Mer* 29**,** 221-229.

Thomas, H. J. & Davidson, C. (1962) The food of the Norway lobster. *Marine Research* 3**,** 1-15.

Thomas, H. J. & Figueiredo, M. J. (1965) Seasonal variations in the catch composition of the Norway lobster, *Nephrops norvegicus* (L.). *Journal du Conseil / Conseil Permanent International pour l'Exploration de la Mer* 30**,** 75-85.

Tocher, D. R. & Harvie, D. G. (1988) Fatty acid compositions of the major phosphoglycerides from fish neural tissues; (n-3) and (n-6) polyunsaturated fatty acids in rainbow trout (*Salmo gairdneri*) and cod (*Gadus morhua*) brains and retinas. *Fish Physiology and Biochemistry* 5**,** 229-239.

Tsape, K., Sinanoglou, V. J. & Miniadis-Meimaroglou, S. (2010) Comparative analysis of the fatty acid and sterol profiles of widely consumed Mediterranean crustacean species. *Food Chemistry* 122**,** 292-299.

Tuck, I., Bailey, N., Atkinson, J. & Marrs, S. (1999) Changes in *Nephrops* density in the Clyde Sea Area, from underwater TV survey data. *Study group on life history of Nephrops. ICES CM 1999/G:13.*

Tuck, I. D., Atkinson, R. J. A. & Chapman, C. J. (2000) Population biology of the Norway lobster, *Nephrops norvegicus* (L.) in the Firth of Clyde, Scotland II: Fecundity and size at onset of sexual maturity. *ICES Journal of Marine Science* 57**,** 1227-1239.

Tuck, I. D., Chapman, C. J. & Atkinson, R. J. A. (1997 a) Population biology of the Norway lobster, *Nephrops norvegicus* (L) in the Firth of Clyde, Scotland I: Growth and density. *ICES Journal of Marine Science* 54**,** 125-135.

Tuck, I. D., Chapman, C. J., Atkinson, R. J. A., Bailey, N. & Smith, R. S. M. (1997 b) A comparison of methods for stock assessment of the Norway lobster, *Nephrops norvegicus*, in the Firth of Clyde. *Fisheries Research* 32**,** 89-100.

Tuck, I. D., Taylor, A. C., Atkinson, R. J. A., Gramitto, M. E. & Smith, C. (1997 c) Biochemical composition of *Nephrops norvegicus*: changes associated with ovary maturation. *Marine Biology* 129**,** 505-511.

Tully, O. & Hillis, J. P. (1995) Causes and spatial scales of variability in population structure of *Nephrops norvegicus* (L.) in the Irish Sea. *Fisheries Research* 21**,** 329-347.

Uglow, R. F., Hosie, D. A., Johnson, I. T. & Macmullen, P. H. (1986) Live handling and transport of crustacean shellfish: an investigation of mortalities. *In: Ministry of Agriculture, F.A.F. (MAFF), Uk, R&D Commission (EDs.), Seafish Technology SR280***,** 24.

Van Ginneken, V. & Van Den Thillart, G. (2009) Metabolic depression in fish measured by direct calorimetry: A review. *Thermochimica Acta* 483**,** 1-7.

Van Weel, P. B. (1974) Hepatopancreas? Comparative Biochemistry and Physiology Part A: Physiology 47**,** 1-9.

Verri, T., Mandal, A., Zilli, L., Bossa, D., Mandal, P. K., Ingrosso, L., Zonno, V., Vilella, S., Ahearn, G. A. & Storelli, C. (2001) D-glucose transport in decapod crustacean hepatopancreas. Comparative Biochemistry and Physiology A-Molecular & Integrative Physiology 130, 585-606.

Watts, A. J. R., Mccafferty, D. J., Newton, J. & Bailey, D. M. (2011) Does seabird carrion contribute to the diet of the shore crab *Carcinus maenas* on the Isle of May, Scotland? An isotopic perspective. *Journal of the Marine Biological Association of the United Kingdom* 91**,** 1459-1464.

Whyte, J. N. C., Englar, J. R., Carswell, B. L. & Medic, K. E. (1986) Influence of starvation and subsequent feeding on body composition and energy reserves in the prawn *Pandalus platyceros*. *Canadian Journal of Fisheries and Aquatic Sciences* 43**,** 1142-1148.

Wieczorek, S. K., Campagnuolo, S., Moore, P. G., Froglia, C., Atkinson, R. J. A., Gramitto, E. M. & Bailey, N. (1999) The composition and fate of discards from *Nephrops* trawling in Scottish and Italian waters. *Final Report to the European Commission. Reference 96/092, Study Project in support of the Common Fisheries Policy* (XIV/96/C75)**,** 323.

Williams, C. T., Buck, C. L., Sears, J. & Kitaysky, A. S. (2007) Effects of nutritional restriction on nitrogen and carbon stable isotopes in growing seabirds. *Oecologia* 153**,** 11-18.

Winget, R. R., Rouse, A. M. & Maurer, D. (1977) Seasonal fluctuations in brachyuran nutrient storage: glycogen in *Callinectes* midgut glands. *Comparative Biochemistry and Physiology A: Physiology*56**,** 69-71.

Withnall, F. (2000) Biology of Yabbies. Aquaculture Notes. www.growfish.com.au/Grow/Files/fn082.pdf

Worden, M. K., Clark, C. M., Conaway, M. & Qadri, S. A. (2006) Temperature dependence of cardiac performance in the lobster *Homarus americanus*. *Journal of Experimental Biology* 209**,** 1024-1034.

Wright, J. C. (2001) Cryptobiosis 300 years on from van Leuwenhoek: what have we learned about Tardigrades? *Zoologischer Anzeiger* 240**,** 563-582.

Yonge, C. M. (1924) Studies on the comparative physiology of digestion II- The mechanism of feeding, digestion, and assimilation in *Nephrops norvegicus*. *The British Journal of Experimental Biology* 1**,**343-389.

Zhang, P., Zhang, X., Li, J. & Gao, T. (2009) Starvation resistance and metabolic response to food deprivation and recovery feeding in *Fenneropenaeus chinensis* juveniles. *Aquaculture International* 17**,**159-172.